



Aspergillus nidulans as a platform for discovery and characterization of complex biosynthetic pathways

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***Aspergillus nidulans* as a platform for
discovery and characterization of complex
biosynthetic pathways**

Diana C. Anyaogu

PhD thesis

Technical University of Denmark

Department of Systemsbiology

Preface

This study was carried out at Systems Biology, Technical University of Denmark, from November 2011 to November 2014 under the main supervision of Professor Uffe Hasbro Mortensen (DTU Systems Biology) and co-supervision by associate professor Mhairi Workman (DTU Systems Biology). The study was supported by DTU and grant 09-064967 from the Danish Council for Independent Research, Technology, and Production Sciences.

Many people have contributed to making the last three years an educational and highly developing time. First and foremost I would like to thank my supervisors, who have always been there for me when I needed help. Thank you for all the guidance and help throughout the years. I am very grateful.

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Finally I would like to thank my family for all the support and understanding they have given me throughout my studies.

Summary

Filamentous fungi produce a wide range of bioactive compounds, classified as secondary metabolites, which have the potential to be used as pharmaceuticals, insecticides, fungicides and food additives. Secondary metabolites also include mycotoxins, which are produced by fungi that contaminate food and feed. Secondary metabolites therefore both have a positive and deleterious impact on the human health. The increase in available genome sequences of fungi has revealed that there is a large number of putative secondary metabolite biosynthetic gene clusters to be discovered and potentially exploited as pharmaceuticals. Access to this unexploited reservoir is hampered as many of the clusters are silent or barely expressed under laboratory conditions. Methods for activating these pathways are therefore essential for pathway discovery and elucidation.

Filamentous fungi and *Aspergillus* species in particular are used in industrial applications for the production of these bioactive compounds and other chemicals as well as for enzyme production. Especially *Aspergillus niger* and *Aspergillus oryzae* are used as industrial workhorses for the production of various enzymes. Many of the secreted proteins are glycosylated, indicating that glycosylation plays an important role in the secretory pathway. Thus, understanding the role and process of glycosylation will enable directed glycoengineering in Aspergilli to improve protein production and expand the repertoire of proteins, which can be produced by these fungi.

Aspergillus nidulans has been used as a model organism for a range of research disciplines and many genetic engineering tools are available for working in this organism. This PhD study therefore employed *A. nidulans* as a model system to address the following on two aspects: 1) Developing *A. nidulans* as a platform for pathway discovery of secondary metabolites and 2) Developing *A. nidulans* as a model system for protein production with human-like glycan structure.

The first part of this study resulted in the development of a method for the transfer and expression of intact biosynthetic gene clusters to *A. nidulans* to facilitate pathway and product discovery. As proof of concept the biosynthetic gene cluster for production of the polyketide geodin was identified and transferred from *A. terreus* to *A. nidulans*. The cluster was integrated in a well characterized locus in *A. nidulans*. Reconstitution of the cluster resulted in the production of geodin. Expression of the enzymes in the pathway was validated by transcription analysis and the functions of specific genes were investigated by gene deletions. This proved that this method is a fast and easy way to transfer biosynthetic gene clusters regardless of size and characterize them. Furthermore, a different approach to activate silent clusters was

demonstrated, as the heterologous expression of a putative transcription factor from *A. niger* in *A. nidulans* induced the synthesis of insect juvenile hormones in *A. nidulans*, which had previously not been reported as fungal metabolites.

The second part of the study focused on understanding the glycosylation pathway in *A. nidulans* and engineering a strain capable of producing precursors for the further modification of the glycan structure towards a more human-like pattern. Previous studies have shown that the deletion of the first mannosyltransferase in the ER (*alg3*) resulted in the accumulation of sizes from $\text{Man}_3\text{GlcNAc}_2$ to $\text{Man}_7\text{GlcNAc}_2$. This study shows that the remaining mannosyltransferases in the ER do not use the truncated structure generated from the *alg3* deletion as a substrate, thus the remaining mannosyltransferase activity must take place in the Golgi. Furthermore, there is an indication that the $\text{Man}_5\text{GlcNAc}_2$ structure generated from the *alg3* deletion is trimmed to $\text{Man}_3\text{GlcNAc}_2$ and extended to $\text{Man}_5\text{GlcNAc}_2$, which has a different structure than the first generated structure by the *alg3* deletion. The work done during this study gives more insight into the glycosylation pathway of *A. nidulans*, which can be used as a basis for further engineering of the pathway to produce humanized glycans in *Aspergilli*.

Sammenfatning

Filamentøse svampe producerer en række bioaktive molekyler, klassificeret som sekundære metabolitter, som har potentialet til at bruges som lægemidler, insekticider, fungicider og tilsætningsstoffer til fødevarer. Sekundære metabolitter omfatter også mykotoksiner, som produceres af svampe, når de kontaminerer fødevarer. Sekundære metabolitter har derfor både en gavnlig og en skadelig påvirkning på menneskers helbred. Den øgede tilgængelighed af svampes genomesekvenser har afsløret, at der er et stort antal af formodede sekundære metabolit biosyntetiske genklustre, som kan opdages og potentielt anvendes som lægemidler. Adgang til denne uudnyttede kilde er hindret af, at mange af disse klustre ikke er udtrykt eller er knapt udtrykt under laboratorie betingelser. Metoder til at aktivere disse synteseveje er derfor vigtige for at opdage og karakterisere ukendte synteseveje.

Filamentøse svampe og især *Aspergillus* arter anvendes i industrien til produktionen af disse bioaktive molekyler og andre kemikaler lige så vel som enzym produktion. Hovedsagelig *Aspergillus niger* og *Aspergillus oryzae* bruges til produktionen af forskellige enzymer. Mange af de proteiner, som udskilles, er glykosyleret, hvilket peger på at glykosyleringen spiller en vigtig rolle i denne proces. At få mere indblik i glykosyleringens rolle og proces vil derfor gøre det muligt at ændre glykosyleringen for at forbedre protein-produktionen og udvide repertoire af proteiner, som kan produceres af aspergilli.

Aspergillus nidulans har været brugt som en modelorganisme i en række forskningsområder, og mange genetiske værktøjer er tilgængelige, når man arbejder i denne organisme. Dette PhD studium har derfor brugt *A. nidulans* som et modelsystem til at tage sig af de følgende to aspekter: 1) At udvikle *A. nidulans* som en platform til at opdage biosynteseveje for sekundære metabolitter og 2) At udvikle *A. nidulans* som modelsystem for produktion af proteiner med de samme sukkerstrukturer, som der findes i mennesker.

Den første del af dette studie resulterede i udviklingen af en metode til at overføre og udtrykke intakte genklustre i *A. nidulans* for at muliggøre opdagelsen af biosynteseveje og produkter. Som et eksempel blev genklustret for biosyntesen af polyketiden geodin identificeret og overført fra *A. terreus* til *A. nidulans*. Genklustret blev integreret i et karakteriseret sted i *A. nidulans* genomet. Dette førte til produktionen af geodin. Ekspressionen af generne i syntesevejen blev eftervist med transskriptionsanalyse, og funktionerne af specifikke gener blev undersøgt vha. gendeletion. Dette beviste, at denne metode er en hurtig og nem måde at overføre genklustre, uanset størrelse, og karakterisere dem. Ydermere blev en anden metode til at aktivere biosynteseveje demonstreret, da heterolog udtryk af en formodet transkriptionsfaktor fra *A. niger* i *A. nidulans* inducerede syntesen af insekt juvenile hormoner, som ikke tidligere var blevet observeret som metabolitter produceret af svampe.

Den anden del af studiet fokuserede på at forstå glykosyleringens biosyntesevej i *A. nidulans* og udvikle en stamme, som er i stand til at producere byggestenen, som kan anvendes til at ændre sukkerstrukturen til et mere humaniseret mønster. Et tidligere studium havde vist, at deletionen af den første mannosyltransferase i ER (*alg3*) resulterede i akkumuleringen af sukkerstruktur svarende størrelse fra $\text{Man}_3\text{GlcNAc}_2$ til $\text{Man}_7\text{GlcNAc}_2$. Dette studie viser, at de resterende mannosyltransferaser i ER ikke anvender den afkortede struktur, genereret af *alg3* deletionen, som et substrat. Den resterende mannosyltransferase aktivitet må derfor finde sted i Golgi. Der er derudover en indikation om, at $\text{Man}_5\text{GlcNAc}_2$, genereret af *alg3* deletionen bliver trimmet til $\text{Man}_3\text{GlcNAc}_2$ og efterfølgende forlænget til $\text{Man}_5\text{GlcNAc}_2$, hvilket har en anden struktur end den første struktur genereret af *alg3* deletionen. Arbejdet udført her giver mere indsigt i glykosylerings syntesevej i *A. nidulans*, hvilket kan bruges som et fundament for yderligere at manipulere syntesevejen til at producere humaniseret sukkerstrukturer i *aspergilli*.

Publications

Peer-reviewed papers

- **Anyao** DC and Mortensen UH. Heterologous production of fungal secondary metabolites in *Aspergilli*. *Frontiers in Microbiology*, *submitted for publication*.
- Nielsen MT*, Nielsen JB*, **Anyao** DC*, Holm DK, Nielsen KF, Larsen TO and Mortensen UH. (2013). Heterologous Reconstitution of the Intact Geodin Gene Cluster in *Aspergillus nidulans* through a Simple and Versatile PCR Based Approach. *PLoS ONE*, 8(8): e72871. *Joint first authors
- Nielsen MT, Klejnstrup ML, Rohlf M, **Anyao** DC, Nielsen JB, Gotfredsen CH, Andersen MR, Hansen BG, Mortensen UH and Larsen TO. (2013) *Aspergillus nidulans* Synthesize Insect Juvenile Hormones upon Expression of a Heterologous Regulatory Protein and in Response to Grazing by *Drosophila melanogaster* Larvae. *PLoS ONE* 8(8): e73369

Conference contributions

- **Anyao** DC, Holm DK, Altintas A, Workman C and Mortensen UH. Development of a high throughput gene expression platform for expression of transcription factors in *Aspergillus nidulans*. Poster presentation at the 12th European Conference On Fungal Genetics. (2014)
- **Anyao** DC, Yang S, Nielsen J B, Zhang H, Betenbaugh M. and Mortensen U.H. N-glycan profiling of *Aspergillus nidulans* using solid-phase glycan extraction and mass spectrometry. Poster presentation at 27th Fungal Genetics Conference, Asilomar, CA, USA (2013). Winner of Poster Award.
- **Anyao** DC, Nielsen MT, Nielsen JB, Hansen BG, Nielsen, KF, Larsen, T O and Mortensen, UH. Heterologous reconstitution of the intact (+)-geodin gene cluster in *Aspergillus nidulans* following a simple and versatile approach. Poster presentation at 7th Danish Conference on Biotechnology and Molecular Biology (2012)

Abbreviations

ACP	Acyl carrier protein
AMA1	Autonomous Maintenance in <i>Aspergillus</i>
Asn	Asparagine
ASPGD	Aspergillus genome database
AT	Acyltransferase
BF	Bright field
BLAST	Basic local alignment search tool
CHO	Chinese hamster ovary
CYC	Cyclase
DH	Dehydratase
Dol-P	Dolichol phosphate
<i>E. coli</i>	<i>Escherichia coli</i>
ER	Enoyl reductase
ER	Endoplasmic reticulum
FAS	Fatty acid synthase
5FOA	5-fluorootic acid
FucT	Fucosyltransferase
Gal	Galactose
GalT	Galactosyltransferase
gDNA	Genomic DNA
GDP	Guanosine diphosphate mannose
GFP	Green fluorescent protein
Glc	Glucose
GlcNAc	N-acetylglucoseamine
GlcNAc-P	N-acetylglucoseamine-phosphate
GnT	N-acetylglucosaminyltransferase
GPI	Glycosylphosphatidylinositol
HR	Highly reducing
HPLC	High performance liquid chromatography
iPKS	Type I iterative PKS
KR	Ketoreductase
KT	β -ketoacyl synthase
Man	Mannose
MAT	Malonyl/acetyltransferase
Mdp	Monodictaphenone
M-Pol	Mannan polymerase
MNS	Mannosidase
MS	Mass Spectrometry
NeuAc	N-acetylneuraminic acid
NeuGc	N-glycolylneuraminic acid

NHEJ	Non-Homologous End-Joining
NR	Nonreducing
NRP	Non-ribosomal peptide
NRPS	Non-ribosomal peptide synthetase
OSMAC	One strain many compounds
OST	Oligosaccharyltransferase
PCR	Polymerase chain reaction
PK	Polyketide
PKS	Polyketide synthase
<i>P. pastoris</i>	<i>Pichia pastoris</i>
PR	Partially reducing
PT	Product template
PTS1	Peroxisomal targeting signal type 1
RFP	Red fluorescent protein
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
Ser	Serine
SiaT	Sialyltransferases
SM	Secondary metabolites
Thr	Threonine
TF	Transcription factor
UDP	Uridine diphosphate
UGGT	UDP-Glc:glycoprotein glucosyltransferase
UPR	Unfolded protein response
USER	Uracil Specific Excision Reagent

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1 Introduction

1.1 Introduction to thesis

Filamentous fungi are used for the production of pharmaceuticals, enzymes and food additives. The ability of fungi to produce bioactive compounds with important activities including antifungal, antibacterial, anticancer and immunosuppressive activities has been exploited since the discovery of penicillin (Fleming, 1929). Genome sequencing has revealed that there is a huge potential of undiscovered secondary metabolites (SMs), as the number of potential SM gene clusters is much higher than the number of characterized gene clusters. Therefore, methods for discovering and activating these pathways are essential for product production and pathway elucidation.

The genus *Aspergillus* contains many important species that are used for industrial production of enzymes and organic acids (e.g. *A. niger*), food production (e.g. *A. oryzae*), but also contain toxin producing food contaminants (e.g. *A. aculeatus*), and aspergillosis (e.g. *A. fumigatus*) as well as the well-studied model organism, *A. nidulans*.

It is important to have an available model system or platform to enable the study of biosynthetic pathways whether in the context of the production of SMs or enzymes. Developing a platform with a set of available genetic tools will facilitate easier pathway elucidation of SMs, either native SM pathways or heterologous expressed SM pathways, or enable the studying of specific pathways involved in the posttranslational modification and secretion of proteins.

The work presented in this thesis has focused on utilizing and developing the model organism *A. nidulans* as a platform for the study of complex biosynthetic pathways. The aim has been two-sided: The first part of the work focused on development of approaches for SM pathway activation and characterization in *A. nidulans* (section 2). The second part focused on expanding *Aspergilli* protein production capacity by engineering the glycosylation pathway (section 3).

The first chapter of this thesis gives a general introduction to filamentous fungi, secondary metabolism and protein glycosylation, which will provide a background for the following chapters. The following chapter presents the results of activating SM pathways in *A. nidulans* and transfer of the geodin cluster from *A. terreus* to *A. nidulans*. Subsequently, the results from the study of the glycosylation pathway in *A. nidulans* is presented.

1.2 Filamentous fungi

Filamentous fungi, or moulds constitute a huge and diverse group of multicellular eukaryotes that are widespread in nature. Fungi have the ability to survive in very diverse environments, can consume and degrade a wide range of substrates and tolerate a range of temperatures and pH. Filamentous fungi are essential to the ecosystem, because of their ability to degrade organic matter, which shows their excellent ability to secrete extracellular enzymes for degradation of complex substrates. Importantly, filamentous fungi produce a wide range of bioactive compounds, which can be exploited to improve human health as e.g. antibiotics and immunosuppressants. Today the production of bioactive compounds from fungi comprises a billion-dollar industry. Unfortunately, filamentous fungi contaminate agricultural crops and are therefore also viewed as food and feed spoilers (Filtenborg et al., 1996) and some fungi are human pathogens (Wüthrich et al., 2012).

1.2.1 *Aspergillus nidulans*

Filamentous fungi within the genus *Aspergillus* have been used for more than 1000 years for brewing and production of fermented food (Machida et al., 2008). Today, they remain important because of their additional role as cell factories for production of primary metabolites and enzymes (*A. niger*, *A. oryzae*) in processes with GRAS (generally recognized as safe) status (Meyer et al., 2011b). Furthermore, *A. nidulans* is a well-studied model organism. On the other hand some Aspergilli are food and feed contaminants (*A. niger*, *A. flavus*).

The establishment of *A. nidulans* as a model organism began with the pioneering work of Guido Pontecorvo in the 1950s within classic genetics (Pontecorvo, 1953). The status as model organism has later expanded to other topics such as cell biology and secondary metabolism (Timberlake, 1988; Morris, 1992) making *A. nidulans* a well-studied model organism for eukaryotes. The early access to the genome sequence of *A. nidulans* (Galagan et al., 2005) has facilitated genome mining in *A. nidulans* (Galagan et al., 2005; David et al., 2008) as well as genome wide deletion studies (Nielsen et al., 2011; De Souza et al., 2013; Brown et al., 2013). As a result several tools for genetic engineering in *A. nidulans* have been developed.

There are a number of attractive features about *A. nidulans*; it grows to high cell density on chemically defined and low-cost medium and has the ability to perform post-translational protein modification. Another attractive feature of *A. nidulans* is the possession of a defined sexual cycle, which allows for strain crossing, as well as an asexual cycle based on unicellular spores. In addition to the sexual and asexual cycle, *A. nidulans* also has a third lifecycle termed the parasexual cycle.

A. nidulans consists of tubular cells, called hyphae, which form a massive interconnected network termed the mycelium (see Figure 1.1). In *Aspergilli*, the hyphae are divided into compartments by septa. However, the septa are perforated walls that allow the passage of nutrients, proteins and organelles (Walther and Wendland, 2003). Secretion of proteins also takes place at the septa in addition to the hyphal tip (Hayakawa et al., 2011).

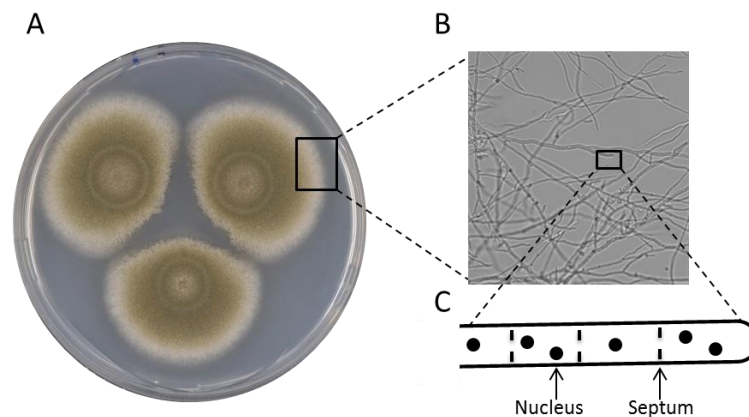


Figure 1.1: Vegetative growth of *A. nidulans*. (A) *A. nidulans* grown on minimal medium at 37°C. (B) The mycelium consists of a network of tubular cells, hyphae. (C) Septa divide the hyphae into compartments.

The asexual life cycle of *A. nidulans* begins with a single unicellular spore called conidium. Under the right conditions the conidium germinates to form a germling, which further develops to form hyphae and form the mycelium (homokaryon). Conidia are produced from specialized aerial structures called conidiophores, which emerge as stalks from the hyphae (Casselton and Zolan, 2002). Hundreds of identical conidia are produced in long chains, and the conidia will thereafter be dispersed into the environment.

1.2.2 Genetic engineering tools in *A. nidulans*

A prerequisite for the establishment of *A. nidulans* as a platform for genetic engineering is the availability of different tools for transformation and selection of transformants, as well as expression of the genes of interest. The selection of correct transformants is greatly facilitated by the use of genetic markers. A number of genetic markers have been described and characterized for use in *A. nidulans* including: *pyrG*, *argB*, *trpC*, *ble*, *hph* (Ballance et al., 1983; John and Peberdy, 1984; Yelton et al., 1984; Cullen et al., 1987; Drocourt et al., 1990; Chiang et al., 2010). Several promoters have also been characterized and used for genetic studies, the most widely used promoters are the constitutive *PgpdA* and the alcohol-inducible *PalcA* (Punt et al., 1991; Waring et al., 1989). Recently, the doxycycline inducible and tunable Tet-on/Tet-off system has been introduced in *Aspergilli* (Meyer et al., 2011a). Adding another promoter to the toolbox.

An important tool that makes strain construction easier in *A. nidulans* was the deletion of *nkuA*, (*ku70*), which is responsible for most of the non-homologous end-joining (NHEJ), which is the most prevalent mechanism responsible for DNA double stranded break repair in *A. nidulans*. The deletion of *nkuA* increased gene targeting efficiency by homologous recombination to > 90% (Nayak et al., 2006; Nielsen et al., 2008), thereby making the construction of strains by gene targeting less cumbersome.

1.2.3 USER™ cloning

Generation of gene-targeting substrates is an important factor in strain construction by genetic engineering. Methods for substrate generation can be divided into two groups: PCR based methods and cloning methods. The major part of the work presented in this thesis was done with Uracil Specific Excision Reagent (USER™) cloning and an introduction to the method will therefore be given. Furthermore, fusion PCR (Erdeniz et al., 1997; Kuwayama et al., 2002; Yu et al., 2004) was used for the generation of deletion mutants in section 3.

The basis of USER™ cloning is the generation of single stranded overhangs by the excision of a single uracil base from a PCR generated fragment. The single stranded overhang will anneal to a complementary overhang. The fragments are transformed into *Escherichia coli* where they are ligated by the native ligase of *E. coli*. Two general approaches are used for generation of USER™ constructs. In one approach the fragments are fused to a PCR amplified vector, while the second approach relies on a vector predigested with restriction enzymes to generate the single stranded overhangs (Nour-Eldin et al., 2006; Geu-Flores et al., 2007). Figure 1.2 illustrates the principle behind the last approach. In this fashion multiple fragments can be fused seamlessly and assembled into large constructs.

Using USER cloning techniques, cloning vectors for gene expression in *A. nidulans* have previously been constructed (Hansen et al., 2011). The vectors are designed for the easy generation of constructs for targeted integration into a defined and characterized site, integration site 1 (*IS1*). The vector contains an expression cassette consisting of a promoter, a selection marker and terminator flanked by targeting sequences for chromosomal integration (see Figure 1.3). The gene of interest is introduced into the vector by USER cloning. After USER cloning correct plasmids are linearized and transformed into *A. nidulans*.

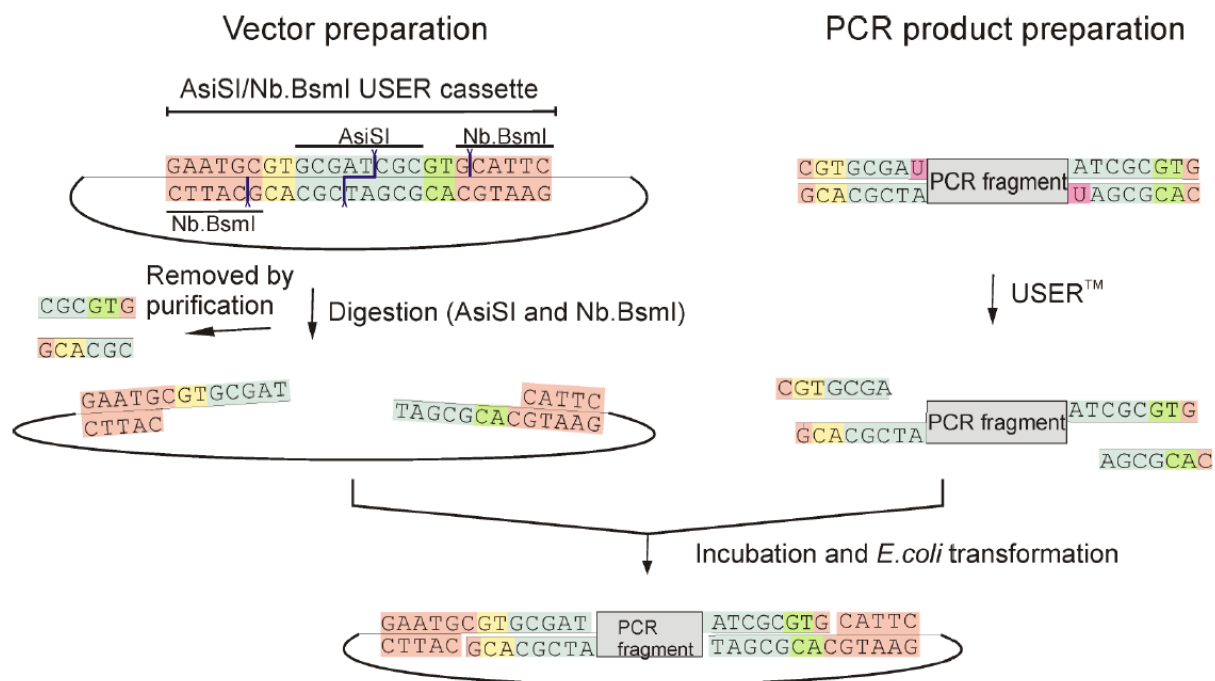


Figure 1.2: Illustrates the principle of USER cloning. The vector is prepared by digestion with restriction enzymes to generate single stranded overhangs. The PCR fragment is amplified by primers containing uracil. The digested vector is mixed with the PCR fragment and USER™. USER™ removes the uracil from the PCR product to generate single stranded overhangs which anneals to the complementary overhangs on the vector. From (Hansen et al., 2011).

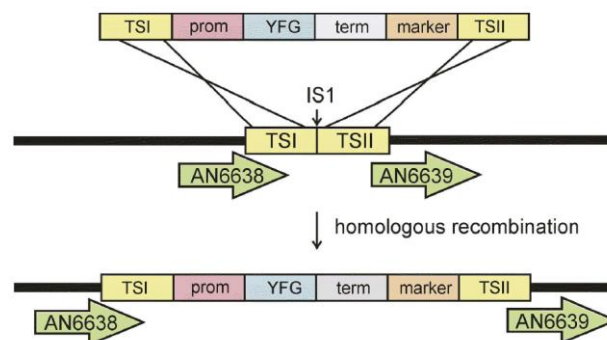


Figure 1.3: Integration of expression cassette into *IS1*. The linearized vector is inserted into *IS1* by homologous recombination. The expression cassette consists of: promoter (prom), YFG (your favorite gene), terminator (term), selection marker and targeting sequences, (TS1 and TSII). From (Hansen et al., 2011)

1.3 Secondary metabolites

Secondary metabolites (SM) are small organic compounds, which are, in contrast to the primary metabolites, not essential for growth and reproduction under non-competitive conditions. However, secondary metabolites provide needed benefits for survival in a competitive environment or non-favorable conditions. Secondary metabolites have various functions in the fungi and have essential roles as differentiation effectors, protection against UV-light (pigments), signal molecules and defense mechanism against competitors (antibiotics, antifungals, insecticides) (Hoffmeister and Keller, 2007). The production of many SM are therefore regulated by stimuli from the environment (Brakhage, 2013). The SMs comprise a range of compounds that are beneficial and deleterious for humans. The beneficial SMs are used as pharmaceuticals (e.g. antibiotics, cholesterol-lowering drugs), food additives and pigments (Campbell and Vederas, 2010; Dufossé et al., 2014). The deleterious effects of SMs are most often due to the mycotoxins produced by the fungi, which are potent carcinogens. In addition to the loss of crops due to the fungal infections (Eaton and Gallagher, 1994; Hussein and Brasel, 2001; Richard, 2007; Voss and Riley, 2013). Because of these impacts on human life it is important to study these compounds.

Three of the major groups of SMs from filamentous fungi, which have interesting biological activities, are; polyketides (PK), non-ribosomal peptides (NRPs)/alkaloids and terpenoids. Furthermore, compounds may also be hybrids and consist of different moieties from the different groups (Klejnstrup et al., 2012). PKs are synthesized by assembly of ketide units by a polyketide synthase (PKS), which is a large multifunctional enzyme similar to the fatty acid synthase (FAS). NRPs are peptides that are synthesized by a non-ribosomal peptide synthetase (NRPS), which are multimodular enzymes. Terpenoids are hydrocarbons made up by C₅ isoprene units (Keller et al., 2005; Marahiel, 2009; Oldfield and Lin, 2012). As the focus of this thesis has mainly been on PKs, a description of the synthesis of PKs will be given in the following section.

1.3.1 Fungal polyketide biosynthesis

PKs are the most abundant fungal SMs, but are also widespread in plants and bacteria (Cox, 2007). PKs are very diverse in structure and function and contain a variety of important biological activities, such as antibacterial, antifungal, anticancer, cholesterol lowering and immunosuppressive properties. Some of the best known fungal PKs are the cholesterol lowering lovastatin (Campbell and Vederas, 2010), the immunosuppressant mycophenolic acid (Bentley, 2000) and the carcinogenic aflatoxin (Eaton and Gallagher, 1994).

The PKSs that synthesize PKs can be classified into three groups based on their primary structure and catalytic mechanisms: type I PKS, type II PKS and type III PKS (Cox, 2007). The type I and type III PKS consist of a large multifunctional multi-domain enzyme and type I PKSs are similar to FAS. In contrast, the type II

PKS is comprised of a system of enzymes with individual domains. The type I PKS can further be subdivided into groups: modular or iterative (Cox and Simpson, 2009). In the modular type I PKS each module contains the set of domains needed for each elongation step and each module is only used once. The growing chain is hereafter transferred to the next module in an ordered fashion (Staunton and Weissman, 2001). In contrast, the iterative PKS only contains a single module, which is used iteratively. Thus, the product of the iterative PKS is not easily predicted. Most of the PKs in fungi are synthesized by type I iterative PKS (iPKS) (Cox and Simpson, 2009).

There are three essential domains for biosynthesis of a PK by the PKS: the acyl carrier protein (ACP) domain, the acyltransferase (AT) domain and the β -ketoacyl synthase (KS) domain (Table 1.1). PKs are usually assembled by repetitive decarboxylative Claisen condensations using acyl-CoA as a starter unit and malonyl-CoA as extender units. AT (or MAT) domain recognizes and transfers the starter unit or extender unit onto the ACP domain, which is responsible for transiently holding the growing PK chain. The KS domain catalyzes the C-C bond formation by Claisen condensations between thioesters (Chiang et al., 2010; Klejnstrup et al., 2012) see Figure 1.4. The type III PKS is an exception as they do not have the ACP domains and condensation is catalyzed by the KS domain (Austin and Noel, 2003; Seshime et al., 2005).

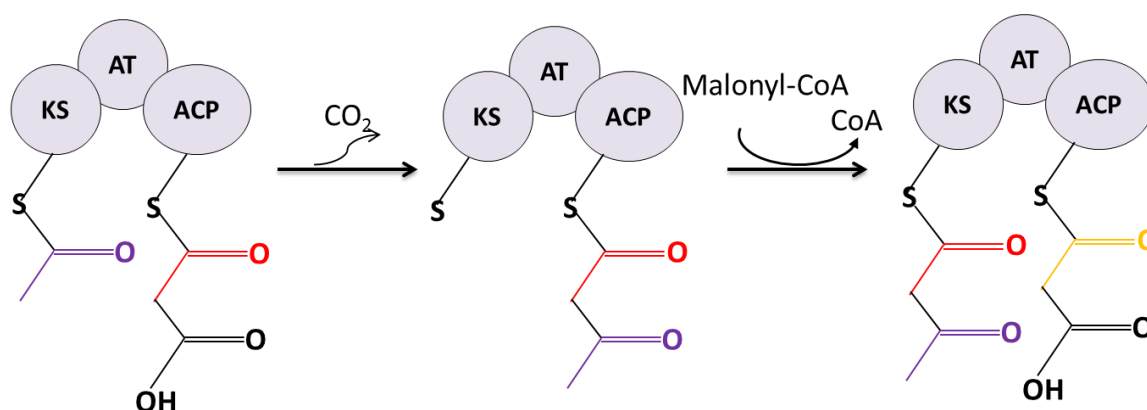


Figure 1.4: Elongation of PK chain. PK chain elongation is initiated by the binding of the starter unit (acetyl –CoA) to the KS domain and loading of an extender unit (malonyl-CoA) onto the ACP. The KS domain catalyzes the condensation reaction extending the acetyl starter unit with one ketide and releasing CO_2 in the process. A new elongation cycle is initiated with the transfer of the growing PK chain to KS and reloading of the ACP domain with an extender unit.

PKSs can have additional domains, which introduce more complexity to the PK (Hertweck, 2009b) (see Table 1.1). Reductive domains, also known as β -keto processing domains, are found in PKSs, which reduce the polyketide backbone. These domains are the ketoreductase (KR), which reduces a ketone group into

hydroxyl group, dehydratase (DH), which dehydrates the hydroxyl group and enoyl reductase (ER), which reduces the enoyl group to yield a saturated alkyl. Fungal iPKSs are classified into three groups according to the presence or absence of these reductive domains: nonreducing (NR) PKS, partially reducing (PR) PKS or highly reducing (HR) PKS (Cox and Simpson, 2009; Hertweck, 2009b). The NR-PKSs do not contain any of the reductive domains, while the reducing PKS contain the ER or ER/DH domains (PR-PKS) or all three domains (HR-PKS). Even though all the reductive domains are present, they might not be used in every iteration. This adds more complexity and diversity to the final PK.

Table 1.1: Overview of the different domains found in PKS

Core domains	Function
β -ketoacyl synthase (KS)	Catalyzes the Claisen condensation reaction
Acyl carrier protein (ACP)	Transiently holds the growing PK chain
Malonyl/acyl transferase (M/AT)	Loading of starter and extender acyl units
Reductive domains	
Ketoreductase (KR)	Reduction of β -ketone groups to hydroxyl groups
Dehydratase (DH)	Reduction of hydroxyl groups to enoyl groups
Enoyl reductase (ER)	Reduction of enoyl groups to alkyl groups
Additional domains	
Methyl transferase (MT)	Add methyl groups to growing PK chain
Thioesterase (TE)	Facilitates release of PK by hydrolysis of the thiolester
Claisen cyclase (CYC)	Catalyzes cyclisation of reduced PK
Product template (PT)	Controls folding pattern of NR-PK
starterunit-ACP transacylase (SAT)	Loading of starter units

The PK backbone can further be modified by the addition of methyl groups to the growing PK chain. This reaction is catalyzed by the methyl transferase (MT) domains, which use S-adenosylmethionin as a methyl donor (Fischbach and Walsh, 2006). Elongation and reduction of the PK chain by addition of ketide units will continue until the final length of the chain has been reached. Subsequently, the PK is released from the PKS in a reaction catalyzed by a thioesterase (TE), cyclase (CYC) or other domains on the PKS or by discrete enzymes (Weissman, 2008; Awakawa et al., 2009; Du and Lou, 2010).

After the release of the PK from the PKS the PK can be post-synthetically modified by tailoring enzymes. This modification can be methylations, reductions, linking the PKs to sugar moieties or terpenoids (Lo et al., 2012), thereby furthering increasing the complexity of the PKs.

1.3.2 Secondary metabolite gene clusters

The genes involved in the biosynthesis of PKs are clustered together in the genome. Besides the gene encoding the PKS and the tailoring enzymes, the cluster might also contain other genes encoding proteins that are necessary for the successful product production. These clusters may contain genes that encode transcription factors that regulate the expression of the gene cluster. Furthermore, genes that confer resistance to the potentially toxic compound or export the compound out of the cell might also be found in the gene cluster.

Genomic sequencing has revealed that there are more gene clusters than known metabolites (Galagan et al., 2005; Andersen et al., 2011; Yaegashi et al., 2014). Many of the gene clusters are silent or barely expressed under standard laboratory conditions making it difficult to link the genes to a product (Hertweck, 2009a). Gene clusters for which the corresponding metabolite are unknown are often called cryptic or orphan clusters (Gross, 2007; Brakhage and Schroeckh, 2011). As a consequence a large number of SMs are currently undiscovered.

Several approaches have been used to activate these silent clusters in the native host. These approaches include the 'one strain many compounds' (OSMAC) strategy (Bode et al., 2002), expression of cluster specific transcription factors (Chiang et al., 2009), deletion and overexpression of global regulators of secondary metabolism (Bok et al., 2006), epigenetic modifiers (Williams et al., 2008) and co-cultivation with microorganisms (Schroeckh et al., 2009; Nützmann, 2011). In this study heterologous expression of a TF was used for product discovery (described in section 2.1)

An alternative to activating a gene cluster in the native host is to transfer single genes or the whole gene cluster to a different host. The following describes the use of heterologous expression of SM genes in *Aspergilli*. In this study a method for transferring a gene cluster was developed and used to transfer a biosynthetic pathway from *A. terreus* to *A. nidulans* (described in section 2.2).

1.3.3 Heterologous expression of fungal secondary metabolites

Heterologous expression can be useful for several reasons. The native host can be difficult to cultivate in the laboratory, hazardous to work with or have no genetic tools available. Transferring the gene or gene cluster of interest to a host, which is easy to cultivate and manipulate genetically, facilitates easier characterization of the product of the gene(s).

Bacteria, yeast and filamentous fungi have been used as host organisms for the heterologous expression of fungal SMs, but far more success has been achieved with filamentous fungi. Hence, filamentous fungi are most often the preferred host. The primary reason is that filamentous fungi are natural producers of a

broad range of SMs, and possess the required enzymes for production of SMs, which bacteria and yeast do not have. Furthermore, in contrast to bacteria filamentous fungi are capable of splicing introns. Moreover, correct protein folding and posttranslational modifications such as glycosylation might not be possible in bacteria. In section 2.3 contains a review of the strategies used for heterologous production of SMs in aspergilli.

Gene prediction is essential for successful expression of genes. Prediction of putative SM genes in a sequenced genome is performed automatically by one or several gene prediction algorithms, which create annotations based on domain predictions and homology search. The automated annotations are followed by manual inspections. In this study, predictions provided by Aspergillus Genome Database (AspGD) (<http://www.aspergillusgenome.org/>) were used for *A. nidulans* and predictions provided by Aspergillus Comparative Sequencing Project database (Broad Institute of Harvard and MIT, <http://www.broadinstitute.org/>) were used for *A. terreus* and *A. niger*.

1.4 Glycosylation

Glycosylation is one of the most prevalent post-translational modifications of proteins and approximately 50% of the known eukaryotic proteins are glycosylated (Apweiler, 1999). Glycosylated proteins, also called glycoproteins, are involved in protein folding, maintaining the protein structure, secretion and enzymatic activity (Helenius, 2001; Helenius and Aebi, 2004; Mitra et al., 2006). Studies in filamentous fungi show that glycosylation has an impact on enzyme stability and activity, and engineering of the glycosylation could be used to improve enzyme stability and activity (Beckham et al., 2012; Chen et al., 2014a). The impact of glycosylation on secretion of glycoproteins in filamentous fungi is not well studied (Nevalainen and Peterson, 2014).

Glycosylation is also important in the production of therapeutic proteins as approximately 70% of the therapeutic proteins are glycosylated (Sethuraman and Stadheim, 2006). Glycosylation is involved in protein stability, ligand binding, immunogenicity and serum half-life (Li and d'Anjou, 2009). Proper production of these proteins are therefore of great importance. Today, the majority of therapeutic non-glycosylated and glycosylated proteins are produced by the bacterium *E. coli* and Chinese hamster ovary (CHO) cells, respectively (Durocher and Butler, 2009; Walsh, 2010). CHO cells and most animal cells produce glycoproteins that differ slightly from human proteins, which can reduce the bioactivity and the serum half-life of the protein and can cause an immune response (Costa et al., 2014). For example the glycoproteins can contain the sialic acid, N-glycolylneuraminic acid (NeuGc), which is not observed in humans that can potentially be immunogenic (Padler-Karavani et al., 2008; Sheeley et al., 1997). Though, this occurs less frequently in CHO cells compared to other animal cells. Production of therapeutic glycoproteins in mammalian cells is costly and tedious, requires utilization of serum and entails a risk of infectious agents. There is therefore an interest in finding alternative production hosts with the machinery to perform post-translational modifications. Some of these systems are plants, yeast, insects, algae, slime mold and bacteria (Betenbaugh et al., 2004; Arya et al., 2008; Valderrama-Rincon et al., 2012; Baker et al., 2013; Specht and Mayfield, 2014; Makhzoum et al., 2014).

Filamentous fungi from the *Aspergillus* species are promising, as they can be cultivated rapidly on inexpensive media and possess the capacity to secrete large amounts of protein (Conesa et al., 2001). This secretion capacity is unmatched in comparison with other eukaryotic expression system as mammalian, yeast and insect cells. However, the fungal glycosylation pattern differs from those of mammalian cells and humans. Thus, the glycoproteins are immunogenic in humans and have limited therapeutic value. The challenge is therefore to eliminate the fungal glycan structure and engineer a pathway that will generate

human-like glycans. The aim of this study is to use *A. nidulans* as a proof of concept for the glycoengineering of fungi.

1.4.1 N-glycosylation

Glycans are most often associated with proteins in three different ways; N- and O- linked glycosylation and glycosylphosphatidylinositol (GPI) anchor. The carbohydrate chains (glycans) are attached to the amide nitrogen of asparagine (Asn) in N-linked glycosylation and attached mainly to the hydroxyl group in serine (Ser) and threonine (Thr) residues in O-linked glycosylation. In GPI anchors a glycolipid is linked to a C-terminal amino acid of a protein. These pathways have two things in common (i) they are initiated at the cytoplasmic face of the endoplasmic reticulum (ER) membrane, while the transfer of the glycan takes place in the ER, (ii) they use dolichol phosphate (Dol-P) derivatives as carriers or intermediates (Orlean, 1992). The N-glycosylation pathway is the most prevalent pathway and this is also the focus of the work performed in this thesis, therefore a description of the pathway is given in the following sections.

1.4.2 Synthesis of N-glycans

The biosynthesis of all eukaryotic N-glycans begins on the cytoplasmic face of the ER membrane with the assembly of a dolichol-linked glycan precursor. The growing precursor is translocated by a flippase into the ER and more sugars are sequentially added until the precursor contains 14 residues. The precursor is subsequently transferred from its dolichol anchor to a protein. The precursor structure is conserved in all eukaryotes (Figure 1.5). The protein bound N-glycan is modified in the ER and Golgi by a series of glycosidases and glycosyltransferases. The maturation of the glycan structure in the Golgi differs depending on the organism.

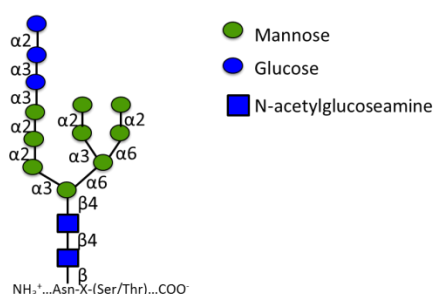


Figure 1.5: Dolichol-linked glycan precursor. This structure is conserved in plants, animals and fungi. The $\alpha x, y$ and $\beta x, y$ denote the type of linkage, and if nothing else is noted x is 1 and is not written, thus $\alpha 3$ and $\beta 6$ correspond to $\alpha 1, 3$ and $\beta 1, 6$.

The pioneering work on the enzymes catalyzing the biosynthesis of N-glycans was performed in the yeast *Saccharomyces cerevisiae*, which is why this pathway and the enzymes characterized in *S. cerevisiae* will be

used to give a general understanding of the N-glycosylation pathway. The synthesis pathway of the N-glycan precursor with the yeast enzymes catalyzing the reactions is illustrated in Figure 1.6.

N-glycan processing in the ER

The synthesis of the common N-glycan precursor begins with the transfer of N-acetylglucosamine-phosphate (GlcNAc-P) from uridine diphosphate (UDP)-GlcNAc to membrane bound Dol-P by GlcNAc-1-phosphotransferase on the cytoplasmic side of the ER. A second GlcNAc residue is added from UDP-GlcNAc and five mannose (Man) residues are transferred stepwise from guanosine diphosphate (GDP)-Man to the precursor by a series of mannosyltransferases. The resulting structure, $\text{Man}_5\text{GlcNAc}_2\text{-P-P-Dol}$ is translocated across the ER membrane by a flippase. Subsequently, four additional mannose residues are added from Dol-P-Man to the glycan core by mannosyltransferases. The transfer of the first mannose residue to $\text{Man}_5\text{GlcNAc}_2\text{-P-P-Dol}$ that generates $\text{Man}_6\text{GlcNAc}_2\text{-P-P-Dol}$ is catalyzed by an α -1,3-mannosyltransferase (Alg3p). The precursor is completed by the transfer of three glucose (Glc) residues from Dol-P-Glc (see Figure 1.6). The sugar donors Dol-P-Man and Dol-P-Glc are generated on the cytoplasmic side of the ER by the transfer of mannose and glucose from GDP-Man and UDP-Glc, respectively, to Dol-P and afterwards flipped across the ER membrane. Dol-P-Glc is generated by glycosyltransferase, while Dol-P-Man is synthesized by a dolichol phosphate mannose synthase, called dolichyl-phosphate- β -D-mannosyltransferase (DPM1p) (Geysens et al., 2009).

The resulting $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2\text{-P-P-Dol}$ is thereafter transferred from the dolichol anchor to a protein being synthesized in ER bound ribosomes. The nascent polypeptide is modified upon entry into the ER by the transfer of the glycan precursor from the dolichol carrier to Asn-X-Ser/Thr (where X can be any amino acid except proline) sequences on the polypeptide. Not all Asn-X-Ser/Thr sequences on the protein become glycosylated due to conformational or other restraints during the process. The transfer of the precursor is catalyzed by a multi-subunit membrane complex termed the oligosaccharyltransferase (OST), which cleaves the GlcNAc-P bond thus also releasing Dol-P-P (Knauer and Lehle, 1999; Kelleher and Gilmore, 2006; Stanley et al., 2009).

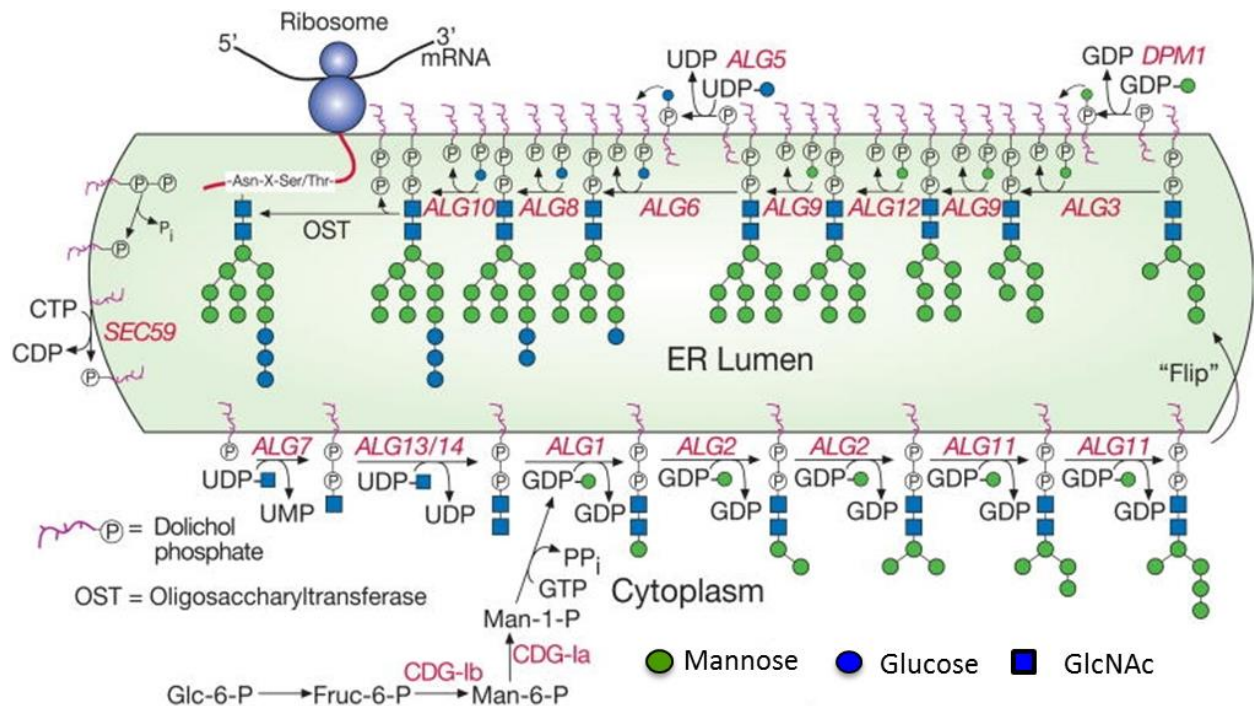


Figure 1.6: Schematic overview of the synthesis of the N-glycan precursor and the transfer of the precursor to a nascent protein. The yeast enzymes catalyzing the reaction are indicated. From (Varki et al., 2009).

After the transfer of the precursor to the protein two glucose residues are removed sequentially by α -glucosidase I and II resulting in a monoglucosylated N-glycan intermediate (Geysens et al., 2009) (see Figure 1.7). The monoglucosylated intermediate is thereafter recognized by molecular chaperons, such as calnexin, or in higher eukaryotes caltreticulin, which assist with the proper folding of the glycoprotein (Hebert et al., 1996, 2005). The glycoprotein is released from the chaperone by deglucosylation of the final glucose residue by α -glucosidase II. If the glycoprotein is incompletely folded, the N-glycans can be reglucosylated by UDP-Glc:glycoprotein glucosyltransferase (UGGT) that acts as a folding sensor within the ER quality control mechanism (Spiro, 2000). The accumulation of unfolded proteins will initiate a response termed the unfolded protein response (UPR). UPR is a way of dealing with ER stress and restore the ER homeostasis. This includes up-regulating the expression of chaperones, foldases and the ER-associated protein degradation by the proteasome (Geysens et al., 2009).

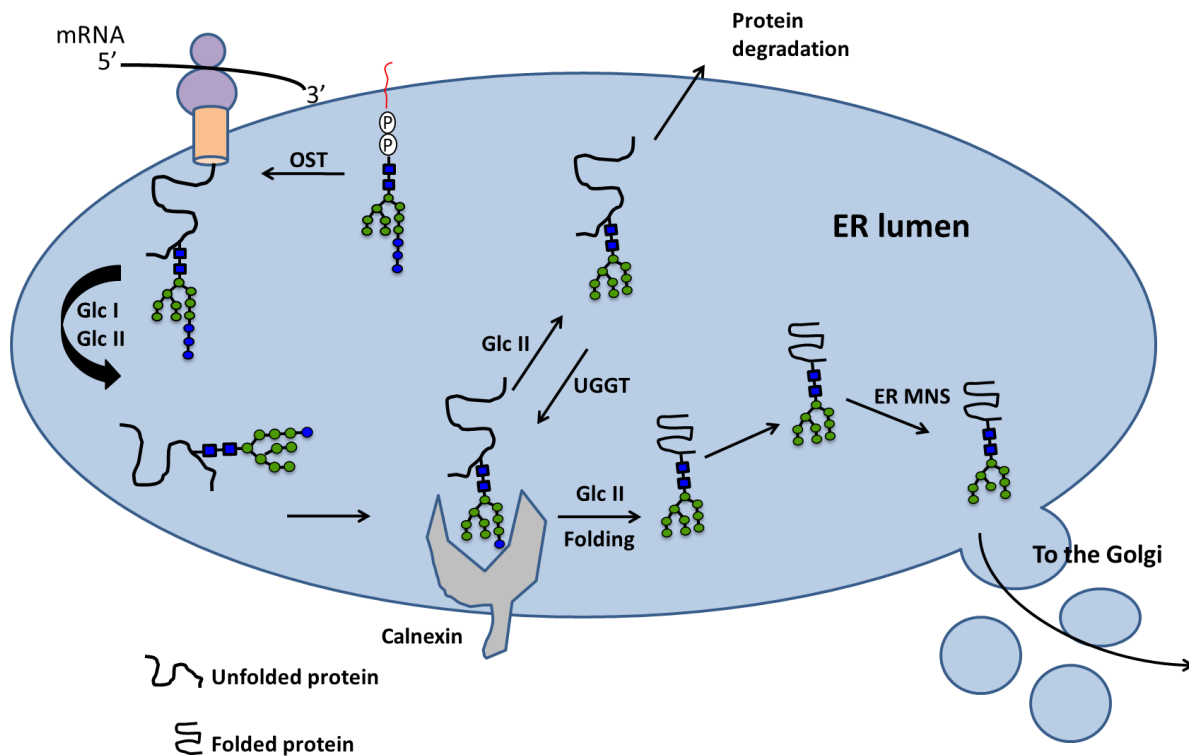


Figure 1.7: Processing of N-glycans in the ER. After the transfer of the precursor to the polypeptide chain the N-glycans are partially deglycosylated by α -glucosidase I and II. The unfolded protein interacts with the chaperone calnexin. The protein is released from calnexin upon removal of the last glucose residue. If the glycoprotein is not folded correctly it may be reglucosylated by UGGT, so that the glycoprotein can interact with calnexin again. If the right conformation is not obtained, the unfolded protein will eventually be sent for degradation. The correctly folded protein is trimmed by ER α -mannosidase and subsequently migrates to the golgi. OST: oligosaccharyltransferase, Glc I and II: α -glucosidase I and II, UGGT: UDP-Glc:glycoprotein glucosyltransferase, ER MNS: ER α -mannosidase I

The N-glycan on many proteins is further trimmed by ER α -mannosidase I or ER α -mannosidase II (ER MNS I or II) to generate $\text{Man}_8\text{GlcNAc}_2$ (Weng and Spiro, 1996) prior to migration from the ER to the Golgi. Due to incomplete processing in the ER some N-glycans consisting of $\text{Glc}_1\text{Man}_9\text{GlcNAc}_2$ may be transported to the Golgi. This structure is the substrate for a Golgi resident endo- α -mannosidase that generates $\text{Man}_8\text{GlcNAc}_2$ by cleaving of the terminal glucose residue and the mannose attached to $\text{Glc}_1\text{Man}_9\text{GlcNAc}_2$ (Varki et al., 2002).

Modification of N-glycans in the Golgi

The glycosylation pathway between yeast and mammals diverge significantly in the Golgi. The activity of Golgi localized glycosyltransferases and glycosidase results in variations among extracellular N-glycans. The branching patterns of N-glycans are classified into three main classes, high-mannose, complex and hybrid

(Kornfeld and Kornfeld, 1985). Figure 1.8 gives an example of each type. The N-glycans from mammalian cells are mainly of the complex type, while the N-glycans from yeast and fungi are commonly of the high mannose type.

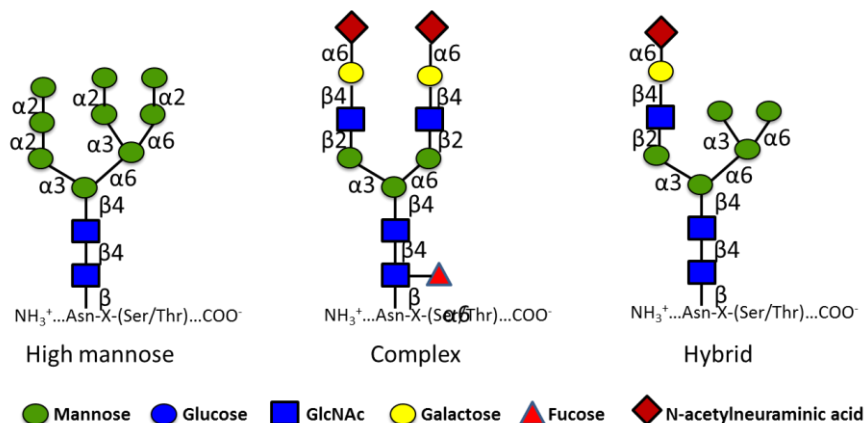


Figure 1.8: Illustration of each class of branching patterns of N-glycans. Adapted from (Varki et al., 2009).

In the Golgi in mammalian cells the $\text{Man}_8\text{GlcNAc}_2$ is trimmed down to $\text{Man}_5\text{GlcNAc}_2$ by three Golgi localized α -1,2-mannosidases, named Golgi α -1,2-mannosidase IA, IB and IC. $\text{Man}_5\text{GlcNAc}_2$ is a key intermediate in the pathway for the generation of hybrid and complex N-glycans (see Figure 1.9). Following the trimming, N-acetylglucosaminyltransferase (GnT) I transfers GlcNAc from UDP-GlcNAc to $\text{Man}_5\text{GlcNAc}_2$. After the transfer of the GlcNAc the majority of $\text{GlcNAcMan}_5\text{GlcNAc}_2$ glycans are further cleaved by α -mannosidase (MNS) II yielding $\text{GlcNAcMan}_3\text{GlcNAc}_2$. The subsequent transfer of a second N-acetylglucosamine residue by N-acetylglucosaminyltransferase II (GnT II) forms the precursor for all biantennary (two branches) complex N-glycans.

Multiantennary N-glycans can be formed by further addition of N-acetylglucosamine residues to the α -1,3-Man and α -1,6-Man by the action of GnT IV, V and VI (see Figure 1.8). N-glycans that are not trimmed by α -mannosidase II and thus still have the $\text{GlcNAcMan}_5\text{GlcNAc}_2$ structure give rise to the formation of hybrid glycans. Similarly, incomplete action of α -mannosidase II can result in $\text{GlcNAcMan}_4\text{GlcNAc}_2$ hybrids. This introduces diversity and complexity in the range of N-glycans synthesized.

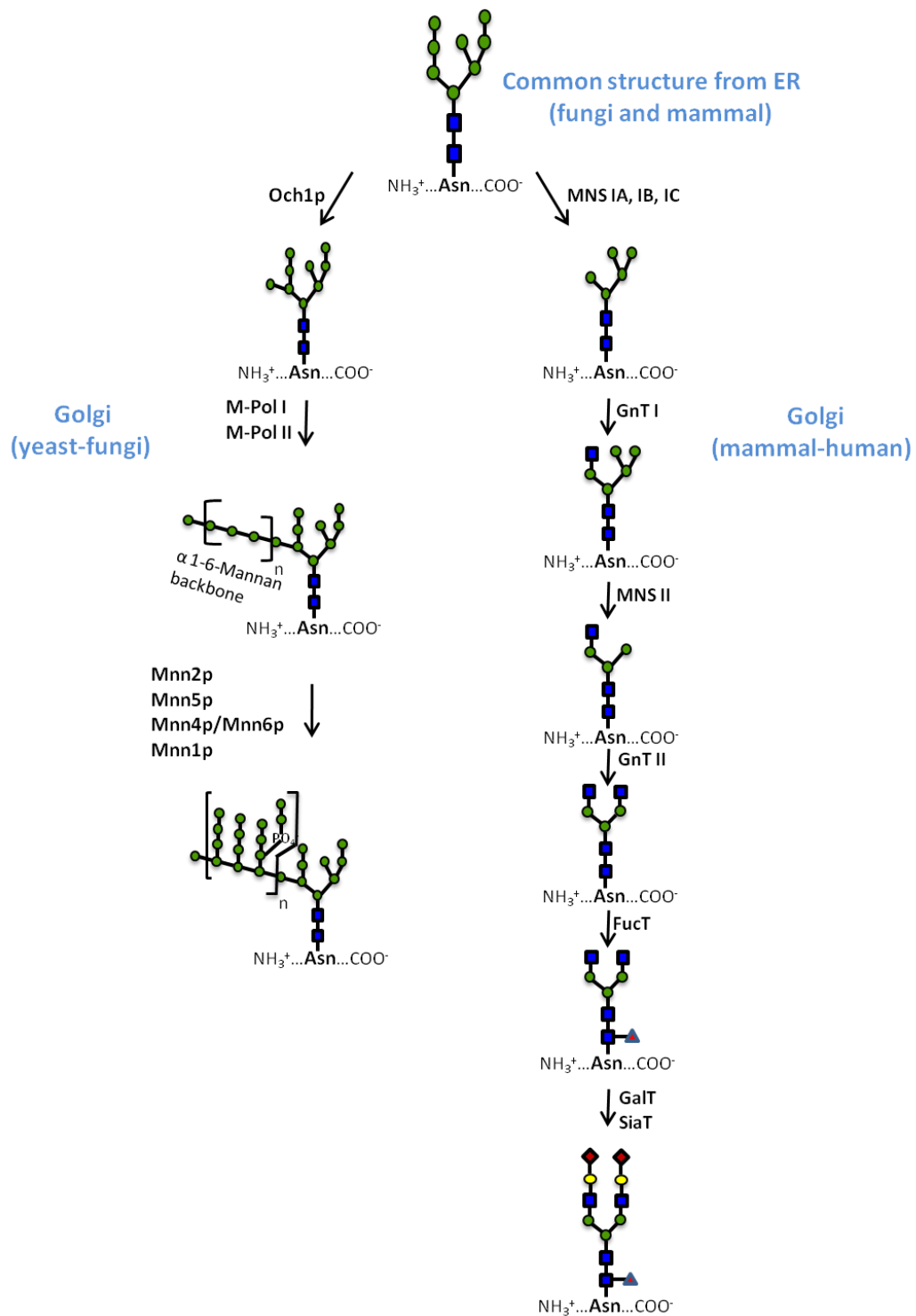


Figure 1.9: Modification of glycans in the Golgi. The N-glycosylation pathway in the Golgi of mammals (right) and yeast (left) resulting in the production of complex (right) and high mannose (left) type glycans, respectively. For more details see text.

The biosynthesis of complex glycans may continue with the transfer of a fucose residue to the N-acetylglucosamine residue adjacent to asparagine in the core. This modification is catalyzed by fucosyltransferase, FucT (Kornfeld and Kornfeld, 1985; Shao et al., 1994). Finally, the N-glycan is extended by the addition of galactose and sialic acid generating the complex N-glycan, which is catalyzed by galactosyltransferase, GalT (Guo et al., 2001) and sialyltransferases, SiaT (Harduin-Lepers et al., 2001), respectively.

In contrast to mammalian cells fungi do not generate complex N-glycans, instead the core oligosaccharide structures are either minimally modified or are highly mannosylated (hyperglycosylated). In *S. cerevisiae*, when the $\text{Man}_8\text{GlcNAc}_2$ structure enters the Golgi elongation of the structure is initiated by the action of an α -1,6-mannosyltransferase, termed Och1p. This enzyme catalyzes the addition of an α -1,6-Man to the structure. N-glycans that are minimally modified will subsequently be modified by mannosyltransferases that add three mannose residues to the structure. In highly mannosylated N-glycans after the action of Och1p up to 50 α -1,6-Man is added to the structure via the sequential activity of two mannosyltransferase complexes, termed complex mannan polymerase (M-Pol) I and II. The α -1,6-Man backbone is further modified by the addition of α -1,2-Man by a number of mannosyltransferases (see Figure 1.9) (Yip et al., 1994; Rayner and Munro, 1998; Geysens et al., 2009).

The N-glycosylation pathway in *Aspergillus* species is not as well characterized as the mammalian pathway or yeast pathway. Deshpande and co-workers used a comparative genomic approach to elucidate the N-glycosylation pathway in *A. nidulans* and *A. niger* (Deshpande et al., 2008). Several orthologous genes from the *S. cerevisiae* hyperglycosylation machinery were found in *A. nidulans* and *A. niger*, and it is thought that they follow the high-mannose pathway just as *S. cerevisiae*, but with a reduced level of glycan mannosylation. Actually, N-glycans found on *Aspergillus* are often small high-mannose structures, while reports of hyperglycosylation in aspergilli are rare (Goto et al., 1997; Maras et al., 1999; Colangelo et al., 1999a, 1999b; Woosley et al., 2006; Qu et al., 2014). Furthermore, some N-linked glycans found in aspergilli are the results of further trimming of the $\text{Man}_8\text{GlcNAc}_2$ structure by mannosidases (Eades and Hintz, 2000; Yoshida et al., 2000).

1.4.3 Glycoengineering in fungi

As previously mentioned the proteins with hyperglycosylated glycans are immunogenic in humans, and the challenge has been to eliminate the hyperglycosylation pathway and introduce the genes from the mammalian N-glycosylation pathway, which are not naturally present in fungi. The first breakthrough was achieved in the yeast, *S. cerevisiae*.

Elimination of the hyperglycosylated structure was achieved by deletion of the *och1* gene, as this gene is responsible for the first step in the hyperglycosylation pathway. The deletion of this gene resulted in the production of glycans with $\text{Man}_8\text{GlcNAc}_2$ structures in yeast (Nakanishi-Shindo et al., 1993). Thus, the majority of subsequent efforts to produce more human-like glycoproteins in fungi are based on this deletion.

In the yeast *Pichia pastoris* several genes have been introduced resulting in the production of the bi-antennary human-like glycan structure, $\text{Sia}_2\text{Gal}_2\text{GlcNAc}_2\text{Man}_3\text{GlcNAc}_2$ (Choi et al., 2003; Hamilton and Gerngross, 2007; Jacobs et al., 2009). The general strategy used to humanize *P. pastoris* is illustrated in Figure 1.10. A suitable precursor required for the synthesis of complex glycans was generated by deleting the *alg3* gene, thus blocking the transfer of mannose to the $\text{Man}_5\text{GlcNAc}_2\text{-P-P-Dol}$ precursor or by the introduction of MNS I to the Golgi. The further introduction of the genes encoding MNSII, GNT I & II, GalT and SiaT together with the precursors needed to produce the substrate utilized by these proteins, generated the complex glycan structure. The glycoengineering of *P. pastoris* has facilitated the production of erythropoietin and IgG with human-like glycan structures (Hamilton et al., 2006; Ha et al., 2011).

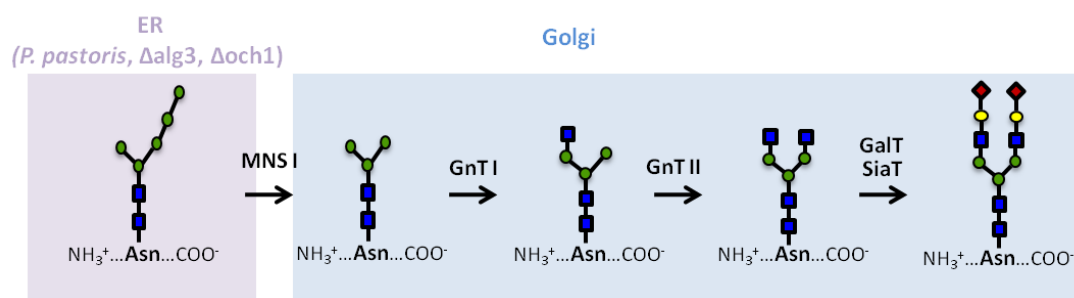


Figure 1.10: Overview of the humanized N-glycosylation pathway in *P. pastoris*. Genes deleted or introduced are indicated on the figure. Adapted from (Hamilton and Gerngross, 2007).

While much progress has been made in the engineering of glycoproteins in yeast, far less has been achieved in filamentous fungi. Several attempts have been made to modify the glycosylation pathway in filamentous fungi (Kalsner et al., 1995; Maras et al., 1999; Kasajima et al., 2006; Kainz et al., 2008).

The most successful approaches were performed by Kainz and co-workers in *A. nidulans* and *A. niger* and were similar to the approach used in *P. pastoris*. The first approach was to introduce a Golgi localized MSN I to generate the $\text{Man}_5\text{GlcNAc}_2$ structure and GnT I to catalyze the addition of GlcNAc. This resulted in the generation $\text{GlcNAcMan}_5\text{GlcNAc}_2$.

The second approach was to delete the *alg3* ortholog (*algC*). They demonstrated that the deletion of *algC* resulted in a shift of the whole glycan pattern to a lower mannose type glycosylation consisting of mainly $\text{Man}_{3-6}\text{GlcNAc}_2$ in *A. niger* and *A. nidulans*. It was also observed that the truncated structure generated by the *algC* deletion, could be further trimmed to $\text{Man}_3\text{GlcNAc}_2$ by the native mannosidases of the fungi. No significant morphological differences or growth defects were observed when the mutant strain was compared to a reference strain. A drawback in this study was the generation of a heterogeneous pool of glycan structures with the deletion of *algC* as well at the presence of $\text{Man}_6\text{GlcNAc}_2$ or higher mannose forms. This limits the available amount of substrate, which can be utilized in the next glycoengineering step, as well as generates glycoproteins with various glycan structure attached, which is undesirable. It is preferable to have a homogenous pool of substrates and section 3.1, describes the work to identify the enzymes responsible for the mannosyltransferase activity and to generate a more homogenous glycan pool.

2 Secondary metabolism

2.1 *Aspergillus nidulans* synthesizes insect juvenile hormones upon expression of a heterologous regulatory protein and in response to grazing by *Drosophila melanogaster* larvae

Being a model organism, *A. nidulans* is very well studied with regards to its secondary metabolism. As mentioned previously in section 1.3 various approaches have been developed and used to identify secondary metabolites. The genome sequencing of *A. nidulans* revealed that the *A. nidulans* genome contains several putative secondary metabolite core genes including 32 putative PKSs (Nielsen et al., 2011), 27 NRPSs and 1 PKS-NRPS hybrid (von Döhren, 2009). By utilizing these approaches several PKS have been linked to products including emodin (Bok et al., 2009), sterigmatocystin (Brown et al., 1996), asperthecin (Szewczyk et al., 2008), asperfuranone (Chiang et al., 2009) and orsellinic acid (Schroeckh et al., 2009) (for reviews see (Kleijnstrup et al., 2012; Yaegashi et al., 2014)). Nevertheless, many genes are still not linked to a product.

As mentioned previously, SMs are not directly required for growth under non-competitive conditions, but play an important role in providing the means for survival under various unfavorable conditions. The production of SMs is regulated to ensure that the SMs are only produced, when they are needed. The regulation of SM biosynthetic genes can occur by TFs. Transcription factors, ranging from pathway specific transcription factors to broad domain transcription factors can transcriptionally control the expression of genes involved in the SM biosynthesis. TFs can either upregulate transcription (activators) or downregulate transcription (repressors). Twelve TF superfamilies have been identified in fungi, with the zinc binuclear (ZN₂Cys₆) family TF being the most abundant group (Shelest, 2008). Most SM cluster specific TFs also belong to the ZN₂Cys₆ family (Yin and Keller, 2011). It has been predicted that *A. nidulans* contains 490 TFs (Wortman et al., 2009), and over 330 of them belong to the ZN₂Cys₆ family. Overexpression of the cluster specific transcription factor has been successfully used to activate silent clusters and link product to genes (Bergmann et al., 2007; Chiang et al., 2009).

The following paper “*Aspergillus nidulans* synthesizes insect juvenile hormones upon expression of a heterologous regulatory protein and in response to grazing by *Drosophila melanogaster* larvae” describes an approach of inducing product production by overexpression of a heterologous TF combined with screening on different media. The expressed TF, which belongs to the ZN₂Cys₆ was transferred from *A. niger* to *A. nidulans*.

Aspergillus nidulans Synthesize Insect Juvenile Hormones upon Expression of a Heterologous Regulatory Protein and in Response to Grazing by *Drosophila melanogaster* Larvae

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Abstract

Secondary metabolites are known to serve a wide range of specialized functions including communication, developmental control and defense. Genome sequencing of several fungal model species revealed that the majority of predicted secondary metabolite related genes are silent in laboratory strains, indicating that fungal secondary metabolites remain an underexplored resource of bioactive molecules. In this study, we combine heterologous expression of regulatory proteins in *Aspergillus nidulans* with systematic variation of growth conditions and observe induced synthesis of insect juvenile hormone-III and methyl farnesoate. Both compounds are sesquiterpenes belonging to the juvenile hormone class. Juvenile hormones regulate developmental and metabolic processes in insects and crustaceans, but have not previously been reported as fungal metabolites. We found that feeding by *Drosophila melanogaster* larvae induced synthesis of juvenile hormone in *A. nidulans* indicating a possible role of juvenile hormone biosynthesis in affecting fungal-insect antagonisms.

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Introduction

Filamentous fungi are capable of synthesizing a wide range of bioactive molecules important for growth and survival in complex and competitive ecological niches [1–3]. A substantial number of these metabolites have been found to have beneficial as well as detrimental impact upon human health. Notable examples of both categories include the pharmaceutically important lovastatin and penicillin [4]; and the mycotoxins fumonisin and aflatoxin that cause health hazards and economical losses when they are present in infected crops [5]. With the release of the full genome sequences of several filamentous fungi it has become apparent that the number of predicted secondary metabolite synthases by far exceeds the number of known metabolites [6,7]. These observations suggest that specific environmental stimuli are required for induction of the majority of secondary metabolites [8]. Despite attempts to identify or mimic these stimuli in order to unravel the secondary metabolism of the model organism *Aspergillus nidulans*,

the product of the majority of predicted synthases are still not known [9,10]. Genetic approaches have been somewhat successful through manipulation of histone methylation [11] or controlled expression of regulatory proteins [12]. As biosynthetic pathways towards secondary metabolites tend to be clustered in the genome [6,7] regulatory proteins likely to be involved in secondary metabolism may be identified by genomic co-localization. However, the number of successful applications of this approach is limited, possibly because far from all predicted gene clusters contain regulatory proteins. We decided to investigate whether induction of secondary metabolites could be achieved through heterologous expression of regulatory genes from other filamentous fungi using the expression of *A. niger* proteins in *A. nidulans* as a test case. A selection of putative pathway specific regulators was tested for this purpose by expressing the corresponding genes individually from a defined locus using a constitutive promoter [13]. This genetic approach was combined with a screen of several complex media recently demonstrated to influence *A. nidulans*

secondary metabolism [14]. This combinatorial approach resulted in the identification of one regulatory protein that strongly induced metabolites not previously reported from *A. nidulans*. Among the induced metabolites were the sesquiterpene hormones methyl farnesoate and insect juvenile hormone-III. Juvenile hormones are required in exact concentrations for correct development of insects and crustaceans [15–17] and therefore hold a strong potential as insecticides [18,19]. To the best of our knowledge, this is the first observation of a fungus with the capacity of synthesizing juvenile hormones. In this manuscript, the biological function of juvenile hormones in *A. nidulans* was addressed through interaction with the saprophagous insect, *Drosophila melanogaster*. We found that when *A. nidulans* was challenged by grazing insects, synthesis of juvenile hormones was induced suggesting that juvenile hormones are part of the fungal defense against invertebrates.

Results and Discussion

Procedure for selection of candidate genes

Selection of candidate regulatory proteins associated with secondary metabolism was based on genomic co-localization of gene clusters. We utilized a collection of previously published microarray experiments from *A. niger* grown under diverse conditions [20–22] to identify regulatory genes associated with predicted secondary metabolite gene clusters using a recently described co-expression based algorithm [23]. Seven candidate genes associated with predicted gene clusters containing either polyketide synthases or non-ribosomal peptide synthases, were identified (Table 1). All seven putative transcription factors belong to the binuclear zinc finger class of proteins, a class often associated with secondary metabolism in fungi [24]. BLAST analysis [25] using the predicted protein sequences against the annotated *A. nidulans* genome (Aspergillus Comparative Database, BROAD Institute) revealed that only one candidate (fge1_pg_C_4000037) had a potential ortholog (ANID_06396, 62% amino acid identity, Table 1). Genes encoding all seven putative regulators were expressed individually in *A. nidulans* under control of the strong constitutive *PgpdA*-promoter from the defined locus *ISI* [13].

Chemical analysis of mutant strains identifies juvenile hormones as metabolites of *A. nidulans*

The resulting mutant strains were grown on minimal glucose media as well as four complex media representing diverse physiological conditions. Metabolite profiles of mycelia extracts were analyzed with liquid chromatography-high resolution mass

spectroscopy (LC-HRMS) as well as ultra-high pressure liquid chromatography diode array detection (UHPLC-DAD) and compared to a reference strain that constitutively transcribes the *E. coli* β -galactosidase-gene (*lacZ*) from *ISI* (NID545). Of all combinations of candidate genes and growth conditions, only *est_fge1_pg_C_150220* (annotation from Aspergillus Comparative Database, BROAD institute of Harvard and MIT) propagated under high salt conditions had an immediately appreciable impact on secondary metabolism resulting in increased accumulation of several metabolites not previously reported to be produced by *A. nidulans* (Figure 1A). Hence, we renamed *est_fge1_pg_C_150220* Secondary Metabolism associated Regulatory protein A (*smrA*). The strain that constitutively transcribe *smrA* was denoted NID477, see Table 2. Correct integration of *smrA* into *ISI*, as well as presence of *smrA* mRNA, was confirmed by Southern blot (Figure 2) and quantitative RT-PCR (Figure 3), respectively. Two induced metabolites displaying very similar UV-spectra were isolated from extracts of NID477 and identified by NMR analysis as the sesquiterpenes: methyl (2E,6E)-10,11-dihydroxy-3,7,11-trimethyl-2,6-dodecadienoate (**1**) (JH-diol) [26] and its formylated analogue (**2**). The formylation, however, was subsequently demonstrated to occur during the extraction procedure and **2** is therefore an artificial derivative of **1**. The sesquiterpene **1** (JH-diol) represents the hydrated form of insect juvenile hormone-III (JH-III). This observation prompted us to search for JH-III and related metabolites using targeted LC-HRMS analysis. Indeed, metabolites with accurate masses corresponding to JH-III and the related crustacean hormone methyl farnesoate (MF) [15] were strongly induced in NID477 compared to the reference, NID545 (Figure 1A). Final identification of these metabolites as JH-III and MF was established by comparison of retention time and mass spectra with an authentic standard (Figure 1B), or with a reference spectra (Xcalibur software package, Thermo Scientific), respectively. The discovery of JH-III and MF as metabolites of *A. nidulans* represents to our knowledge the first report of production of invertebrate juvenile hormones in fungi.

Biosynthesis of JH-III, JH-diol and MF in *A. nidulans*

Biosynthesis of juvenile hormones is well characterized in insects [27]. Since further elucidation of the potential role of juvenile hormones in fungal-insect antagonisms would benefit substantially from the generation of null mutants, we attempted several homology based strategies for identification of the biosynthetic pathway for juvenile hormones in *A. nidulans*. Initially, BLAST analysis was performed using previously characterized insect enzymes as input, however, no obvious candidates were identified

Table 1. Candidate genes from *A. niger*.

Strain #	Broad annotation	Transcript ID	Candidate <i>A. nidulans</i> homologues	Identity percentage
NID357	fge1_pg_C_4000037	38716	ANID_06396, ANID_03269	62%, 27%
NID358	e_gw1_4.316	178503	ANID_07346	26%
NID360	e_gw1_11.945	188323	ANID_08894	25%
NID366	gw1_10.247	123782	None	–
NID367	fge1_pg_C_19000192	45823	ANID_11683, ANID_07921	43%, 22%
NID476	e_gw1_8.296	184613	ANID_04485	30%
NID477	est_fge1_pg_C_150220	54836	None	–

Candidate genes were selected based on co-localization with predicted gene clusters in *A. niger* containing either a polyketide synthase, a non-ribosomal peptide synthase or both. Transcript ID = Annotation from the DOE Joint Genome Institute (genome.jgi-psf.org), candidate *A. nidulans* homologues = Highest scoring potential homologs in *A. nidulans*, Identity percentage = amino acid identity percentage.

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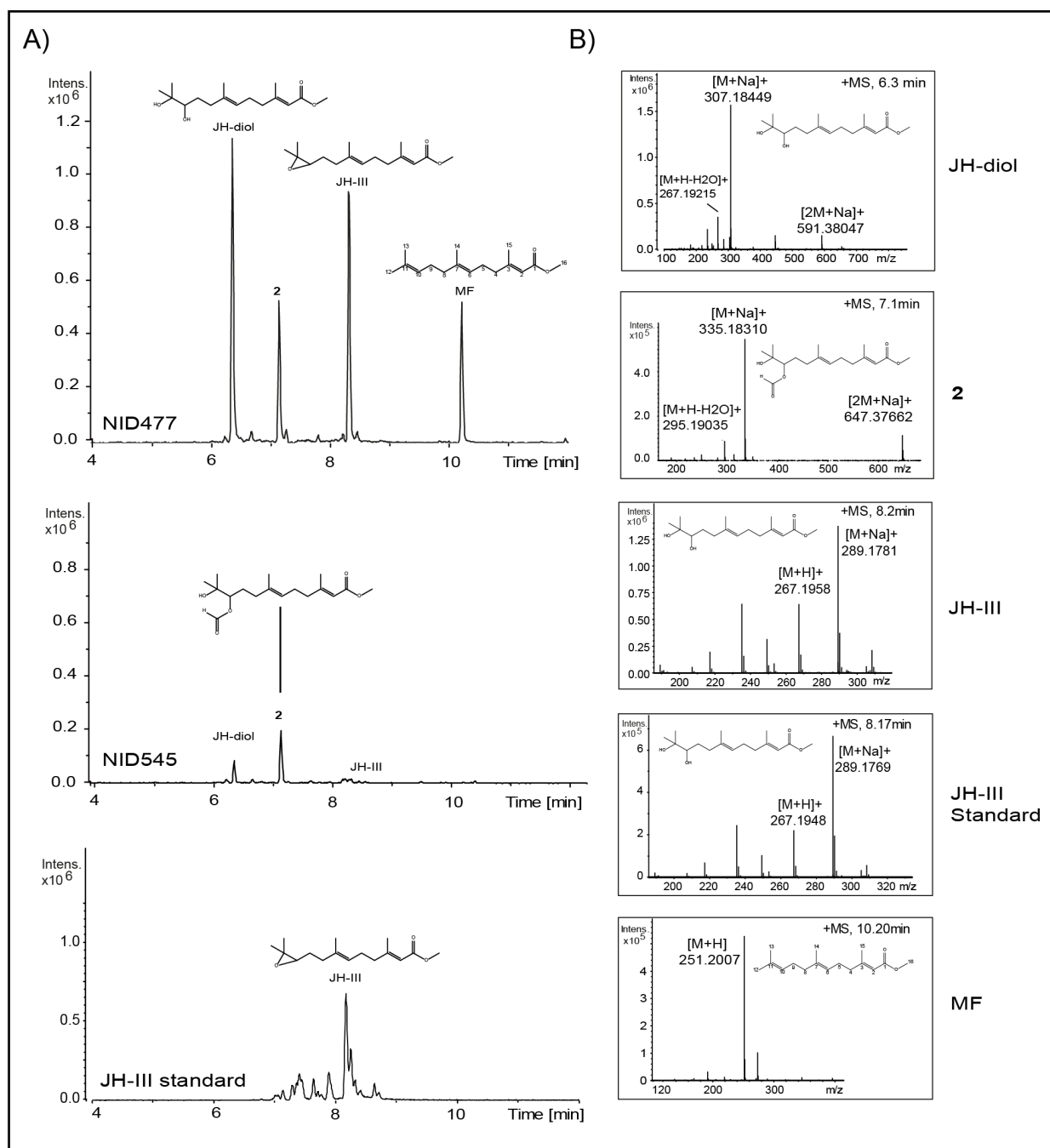


Figure 1. Induction of metabolites by SmrA. A) UHPLC-QTOFMS extracted ion chromatogram of m/z 251 (MF, $[M+H]^+$), 289 (JH-III, $[M+Na]^+$), 307 (JH-diol, $[M+H]^+$) and 335 (2, $[M+H]^+$) recorded in positive mode of extracts from the strain constitutively expressing *smrA* (top) and reference (middle) grown under high salt conditions. Chromatograms are normalized by intensity. Chemical structures of JH-diol, compound 2, JH-III and MF are embedded above the corresponding signal peaks. Bottom panel depicts extracted ion chromatogram of m/z 289 (JH-III, $[M+Na]^+$) of an authentic JH-III standard (65% pure) purchased from Sigma Aldrich. Note that the standard contains several impurities. Panel B): Corresponding mass spectra of JH-diol, compound 2, JH-III and MF in the mutant strain constitutively expressing *smrA* as well as the authentic JH-III standard. Chemical structure of the corresponding molecule is embedded in each panel. doi:10.1371/journal.pone.0073369.g001

(data not shown). We speculate that the long evolutionary distance between insects and *A. nidulans* may have obscured a common origin, but it cannot be excluded that an alternative biosynthetic mechanism has evolved in fungi. We tested whether the mixed PKS/NRPS gene cluster in which *smrA* is located (*A. niger* transcript ID: 192362, 128601, 191998, 44877, 44878, 44880

and 54837) is conserved in *A. nidulans* and could provide an alternative biosynthetic route, however, the cluster is not present in *A. nidulans* as evidenced by BLAST analysis of individual genes (data not shown). Moreover, *SmrA* does not have any homologs in *A. nidulans* (Table 1). Thus homology based methods seems to be challenging. We expect that microarray based analysis of the

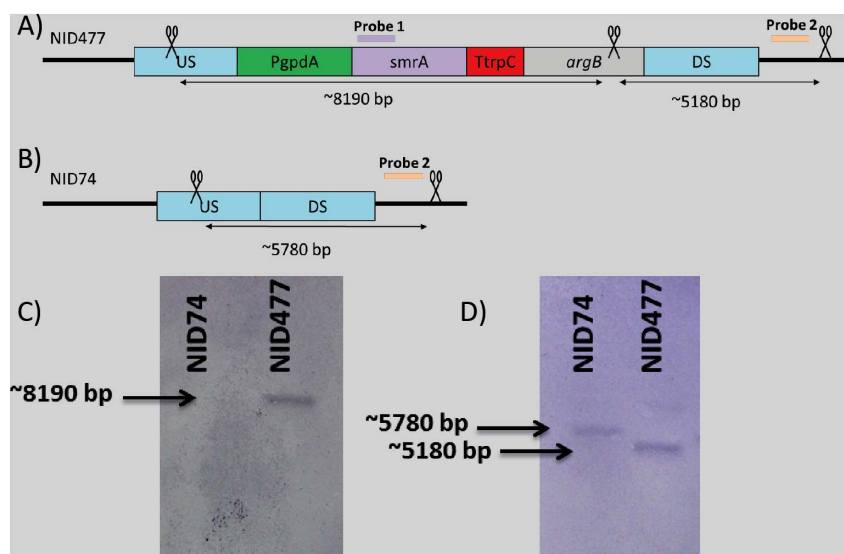


Figure 2. Confirmation of correct integration of *smrA* in IS1. A) and B): Schematic overview of the HindIII cut sites (indicated with scissors) and the size of the resulting fragments. Purple and orange bars indicate hybridization site for *smrA* probe and locus probe respectively. C): Illustration showing placement of the bands relative to each other. D): Southern blot of NID74 and NID477 digested with HindIII and hybridized with *smrA* probe. E): Southern blot of NID74 and NID477 digested with HindIII and hybridized with locus probe. The illustration is not drawn to scale. doi:10.1371/journal.pone.0073369.g002

growth condition dependent synthesis of juvenile hormones in NID477 may serve as a more fruitful route for identification of the juvenile hormone synthesis pathway in *A. nidulans*.

Biological function of Juvenile hormones in *A. nidulans*

Fungal secondary metabolites are known to play an important role in fungal-insect interactions [2,3]. Moreover, the role of juvenile hormones in regulating processes of insect metamorphosis, reproduction and metabolism are well described [16,17]. We therefore hypothesized that the biological function of juvenile hormones in *A. nidulans* is related to interaction with insects. It is known, that timing and dosage of insect exposure to juvenile hormones is crucial for correct development, with fatal consequences of both under- and overexposure [16]. Consequently, synthesis of JH and MF could be employed as a defense mechanism in *A. nidulans* and such a strategy has been demonstrated for the plant *Cyprus iria* [28]. We pursued two experimental lines of evidence in order to test our hypothesis; 1) analysis of the spatial distribution of JH and MF and 2) conducted confrontation experiments between *A. nidulans* and larvae of the saprophagous insect *Drosophila melanogaster*.

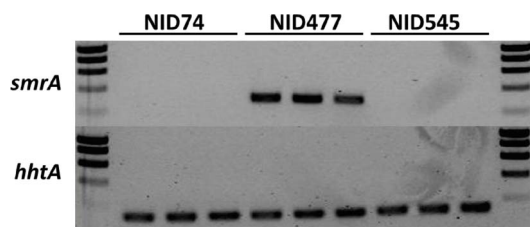


Figure 3. Expression of *smrA* in NID477 and two control strains. Measuring the expression of *smrA* and the control *hhtA* after cultivating the strains on both MM and CYAs gave the same result. No expression of *smrA* could be detected in the controls (top panel), but only in the NID477 strain. doi:10.1371/journal.pone.0073369.g003

Distribution of JH-III, JH-diol and MF

The metabolite composition of growth media extracts and collected volatiles of NID477 and the reference, NID545, grown under juvenile hormone stimulating conditions was analyzed by LC-HRMS and gas chromatography mass spectroscopy (GC-MS), respectively. None of the three terpenes were detectable as extracellular metabolites in the growth media. JH-III and JH-diol were also undetectable among the volatiles whereas MF constituted a major metabolite in the volatile fraction of both strains (Figure 4). Taken together with the presence of JH-III and JH-diol in mycelia extracts (see above), we conclude that JH-III and JH-diol are maintained intracellularly in the mycelium. Therefore, insects will ingest juvenile hormones upon foraging on *A. nidulans* which may disturb the careful balance of juvenile hormone dosage.

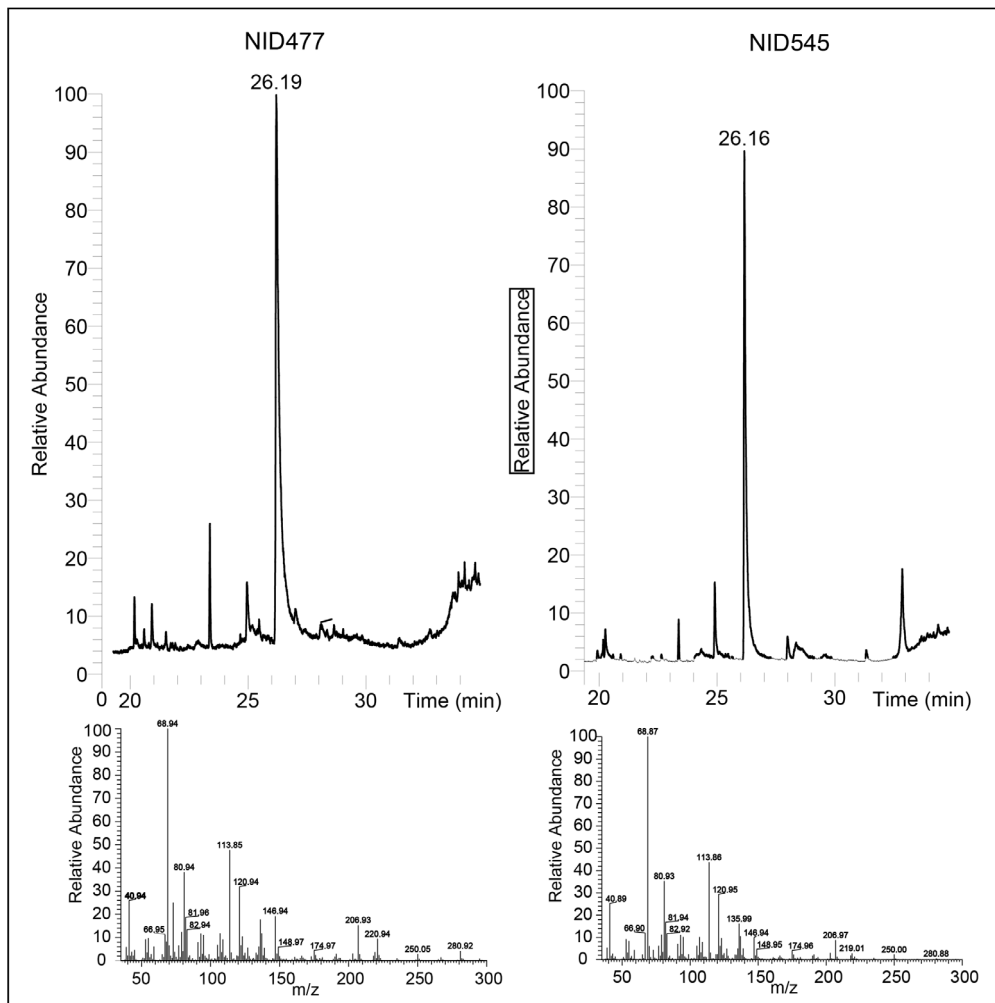
D. melanogaster larvae induce JH-III synthesis upon grazing

D. melanogaster was chosen for the confrontation experiments since the versatile role of juvenile hormones in *D. melanogaster* development is well documented [29] and since patterns of interaction between *A. nidulans* and *D. melanogaster* larvae have been described previously [30]. The confrontation experiments were initially performed under the conditions where *SmrA* stimulated JH-III and MF synthesis. However, the high salt content in the media (5% NaCl) caused severe larval mortality even in mock free controls (data not shown). We therefore decided to perform the experiments under less stressful conditions (standard *Drosophila* medium, [30]). In this experiment, the fitness of grazing *D. melanogaster* larvae was not significantly different between NID545 and NID477 on two of three parameters evaluated (Figure 5). However, flies emerging from the NID477 treatment displayed a significant decreased dry weight, indicating a negative impact of NID477 on *D. melanogaster* fitness compared to NID545. We therefore performed a metabolite analysis of fungal extracts produced from the two strains in the presence or absence of larvae in order to correlate the observed effect with differences in the

Table 2. Name and description of fungal strains used in this work.

Strain #	Genotype	Description	Reference
NID74	<i>argBΔ, pyrG89, veA1, nkuAΔ</i>	Parental strain with permanent deletion of <i>nkuA</i> and <i>argB</i> to facilitate gene targeting	This study
NID545	<i>argBΔ, pyrG89, veA1, nkuAΔ, IS1::PgpA-lacZ::argB</i>	Reference strain with <i>E.coli lacZ</i> integrated in <i>IS1</i> .	This study
NID357	<i>argBΔ, pyrG89, veA1, nkuAΔ, IS1:PgpA:fge1_pg_C_4000037::argB</i>	Constitutive expression of putative binuclear zinc finger transcription factor <i>fge1_pg_C_4000037</i> integrated in <i>IS1</i>	This study
NID358	<i>argBΔ, pyrG89, veA1, nkuAΔ, IS1:PgpA:e_gw1_4.316::argB</i>	Constitutive expression of putative binuclear zinc finger transcription factor <i>e_gw1_4.316</i> integrated in <i>IS1</i>	This study
NID360	<i>argBΔ, pyrG89, veA1, nkuAΔ, IS1:PgpA:e_gw1_11.945::argB</i>	Constitutive expression of putative binuclear zinc finger transcription factor <i>e_gw1_11.945</i> integrated in <i>IS1</i>	This study
NID366	<i>argBΔ, pyrG89, veA1, nkuAΔ, IS1:PgpA:gw1_10.247::argB</i>	Constitutive expression of putative binuclear zinc finger transcription factor <i>gw1_10.247</i> integrated in <i>IS1</i>	This study
NID367	<i>argBΔ, pyrG89, veA1, nkuAΔ, IS1:PgpA:fge1_pg_C_19000192::argB</i>	Constitutive expression of putative binuclear zinc finger transcription factor <i>fge1_pg_C_19000192</i> integrated in <i>IS1</i>	This study
NID476	<i>argBΔ, pyrG89, veA1, nkuAΔ, IS1:PgpA:e_gw1_8.296::argB</i>	Constitutive expression of putative binuclear zinc finger transcription factor <i>e_gw1_8.296</i> integrated in <i>IS1</i>	This study
NID477	<i>argBΔ, pyrG89, veA1, nkuAΔ, IS1:PgpA::smrA::argB</i>	Constitutive expression of <i>smrA</i> (<i>est_fge1_pg_C_150220</i>) integrated in <i>IS1</i>	This study

doi:10.1371/journal.pone.0073369.t002

**Figure 4. Excretion of MF by *A. nidulans*.** Top panel: Total MS chromatogram of the collected volatiles from the *smrA* expressing strain and the reference. Bottom panel: Mass spectrum of the compound eluting at 26.19 minutes. The compound was identified as MF by comparison to the metabolite library of the Xcalibur software package (Thermo Scientific).

doi:10.1371/journal.pone.0073369.g004

metabolite profile. When NID545 and NID477 were grown on standard *Drosophila* medium most of the detectable secondary metabolites (austinol, dehydroaustinol, nidulanin A and sterigmatocystin) did not differ significantly between the two strains (Figure 6A). Importantly, JH-III and the JH-diol were detected in both strains, but was not significantly induced in NID477 on this medium (Figure 6B). These observations are in agreement with the results obtained in the initial screening of the NID477 strain, which revealed that the induction of secondary metabolites was highly condition dependent. Interestingly, comparison of the metabolite profiles obtained from NID477 and NID545 strains grown in the presence or in the absence of grazing *D. melanogaster* larvae demonstrated that the presence of the insects significantly increased the level of both JH-III and JH-diol irrespective of the strain background (p-values JH-III; 0,0288 and 0,00723 and JH-diol; 0,0006 and 0,02415 for NID477 and NID545, respectively, Figure 6C). Curiously, JH-III accumulated to higher levels in NID545 than in NID477 (p-value <0,025). Perhaps, this reflects that when the natural induction of JH-III takes place, the contribution from the presence of the heterologous transcription factor SmrA is detrimental to JH-III biosynthesis. A simple model could be that the natural *A. nidulans* transcription factor and SmrA bind in a competitive manner to the promoters of the genes involved in JH-III biosynthesis and that activation is less efficient when SmrA is present. We consider it likely that constitutive expression of the SmrA transcription factor has numerous other effects on *A. nidulans* that is not reflected in our metabolite analysis, and that collectively these effects cause the observed decrease in *D. melanogaster* fitness. However, the induction of juvenile hormones upon insect feeding, taken together with the well-established involvement of juvenile hormones in insect development and physiology, strongly suggest that JH-III do impact the relation between insects and *A. nidulans*.

Perspectives

The findings of this manuscript indicate that juvenile hormones represent previously overlooked compounds in chemical interactions between *A. nidulans* and insects. In addition, the ability of *A.*

nidulans to synthesize juvenile hormones provides the potential for a bio-based source for juvenile hormone production in cell factories. Juvenile hormones are considered to be among the most potent and promising insecticides due to their high specificity and efficiency [18,19]. Moreover, as *A. nidulans* releases the juvenile hormone MF to the environment, downstream purification of MF would be simple, as MF could be collected from the volatiles as described previously for other sesquiterpenes [31]. Finally, the findings in this manuscript underline how manipulation of regulatory proteins, systematic variation of physical parameters as well as insect-fungus confrontation systems may be valuable tools for modifying fungal secondary metabolite profiles. The latter approach has the advantage of providing clues to biological function of metabolites. A similar approach simulating bacterial-fungal interactions has previously been successful in identifying novel metabolites in *A. nidulans* [32] indicating that this more biological approach may constitute a promising route for future studies.

Materials and Methods

Strains and media

Escherichia coli strain DH5 α was used to propagate all plasmids. All *A. niger* genes were amplified from strain ATCC1015. The *A. nidulans* strain NID74 (*argBA*, *veA1*, *pyrG89*, *nkuA4*) was used as background strain for all transformations as it allows gene targeting with the *argB* marker due to a complete deletion of the *A. nidulans* *argB*-open reading frame. NID74 was generated from NID1 (*argB2*, *veA1*, *pyrG89*, *nkuA4*) using the fusion PCR technique essentially as described previously [33]. NID545 (*argBA*, *pyrG89*, *veA1*, *nkuA4*, *IS1::P_{gpdA}-lacZ-T_{trpC}::argB*) was used as reference strain for metabolite analysis. Genotypes of all strains are summarized in Table 2. All *A. nidulans* strains were propagated on solid glucose minimal medium (MM) prepared as described by Cove [34], but with 1% glucose, 10 mM NaNO₃ and 2% agar. MM was supplemented with 10 mM uridine (Uri), 10 mM uracil (Ura), where required. Complex media used for chemical analysis

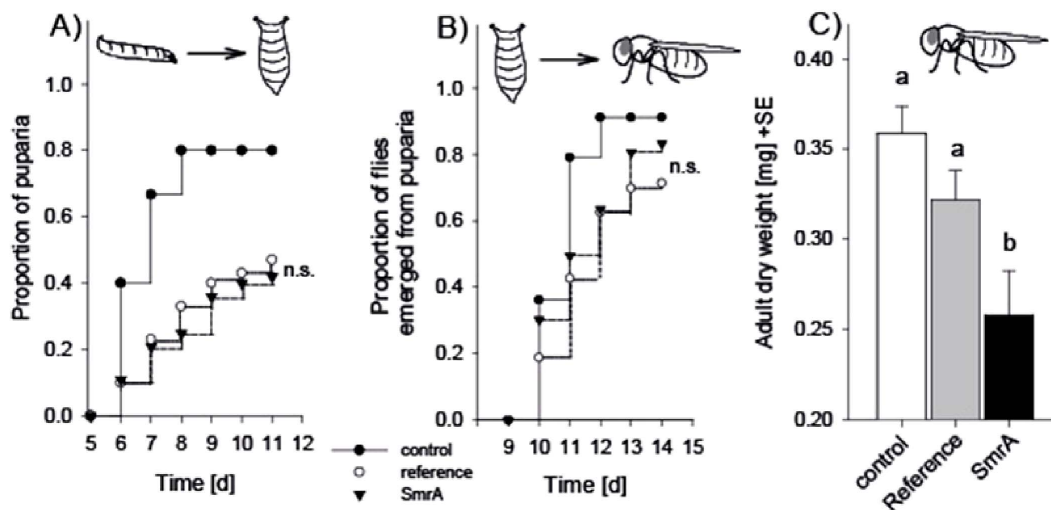


Figure 5. Influence on *D. melanogaster* larva-to-adult development. Panel A): Proportion of *D. melanogaster* larvae that reached the pupal stage as a function of fungal treatment (mold-free control, NID545 or NID477) and time. Panel B): Proportion of flies that emerged from puparia as a function of fungal treatment and time. Panel C): Dry weight of emerged flies as a function of fungal treatment. Different letters indicate statistically significant differences between treatment following a one-way Analysis of Variance ($F_{2,38} = 6.652$, $p = 0.003$) and Holm-Sidak pair-wise comparison. n.s. not significant.

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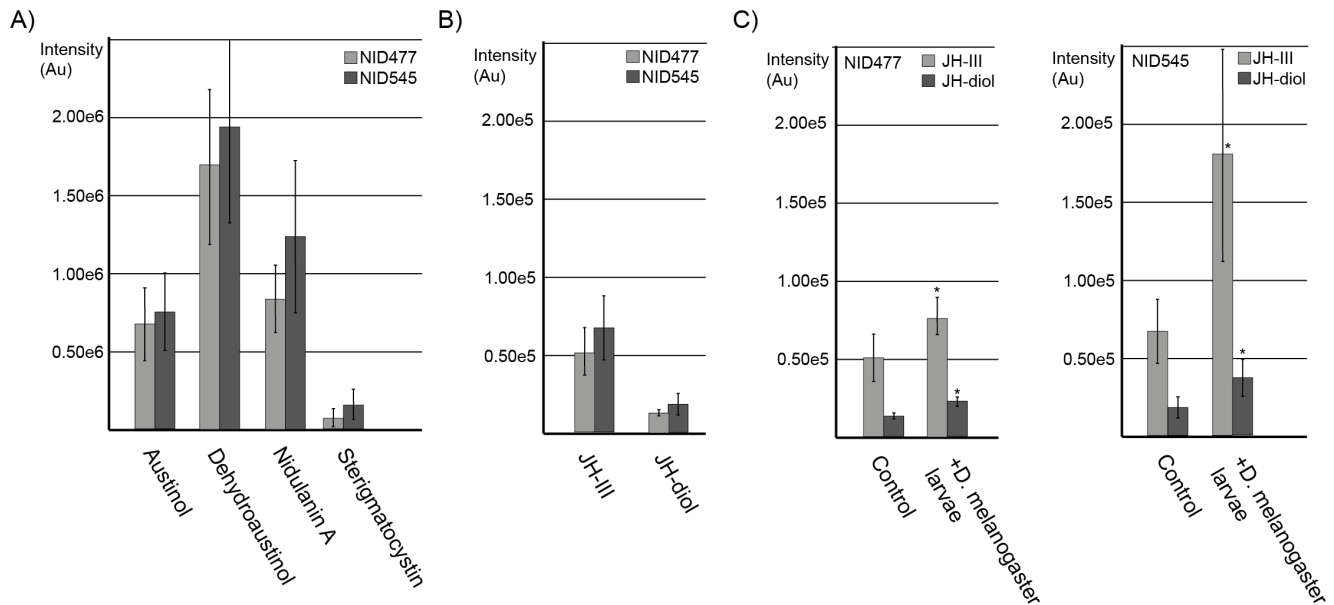


Figure 6. Insect grazing induced alterations of secondary metabolites in *A. nidulans*. Quantification of secondary metabolites: JH-III, JH-diol, austinol, dehydroaustinol, nidulanin A and sterigmatocystin from LC-HRMS analysis. For each metabolite, columns display the average and error bars the standard deviation. Statistical analysis was performed with pair-wise comparisons using the student's t-test. Panel A) comparison of austinol, dehydroaustinol, nidulanin A and sterigmatocystin levels in NID477 and NID545. Panel B) comparison of JH-III and JH-diol levels in NID545 and NID477. Panel C) *D. melanogaster* feeding significantly increases accumulation of JH-III (p-values; 0,0288 and 0,00723) and JH- diol (p-values; 0,0006 and 0,02415) in NID477 and NID545, respectively. doi:10.1371/journal.pone.0073369.g006

were prepared as described by Frisvad and Samson [35] and supplemented with 10 mM uridine and 10 mM uracil.

PCR, USER cloning and *A. nidulans* strain construction

USER cloning compatible PCR products were amplified with 30 PCR cycles in 50 μ l reaction mixtures using proof-reading PfuX7 polymerase [36]. USER vectors were denoted according to the nomenclature introduced by Hansen et al [13]. Putative *A. niger* genes were amplified from *A. niger* genomic DNA, USER cloned into pU1111-*IS1*, and transformed into *A. nidulans* as described previously [13]. In order to generate the NID545 reference strain, the *E. coli lacZ* gene was cloned into a pU1014-*IS1* vector generating pU1011-*IS1:lacZ* which was transformed to a pU1110-*IS1-lacZ* vector by insertion of *A. nidulans gpdA* promoter in the *AsiSI/Nb.BtsI* cassette. All expression plasmids were verified by sequencing. Gene targeting events were verified in all *A. nidulans* transformants by analytical PCR as described previously [13]. Table 3 summarizes the PCR primers used in this study. In addition, NID477 was confirmed by Southern blotting as described in [37]. For each Southern blot 2 μ g genomic DNA was digested with HindIII. Two probes for detecting insertion of the *smrA* gene into *IS1* were generated by PCR. Specifically, primers JBN X66 and JBN X67 were used to generate Probe 1, a 896 bp fragment of *smrA* using genomic DNA from *A. niger* as template, and primers JBN X64 and JBN X65 were used to generate Probe 2, a 948 bp fragment at the *IS1* locus using genomic DNA from *A. nidulans* as template, see Figure 2. The probes were labeled with Biotin-11-dUTP using the Biotin DecaLabelTM DNA Labeling kit (Fermentas). Detection was performed with the Biotin Chromogenic detection kit (Thermo scientific).

RNA isolation and quantitative RT-PCR

RNA isolation from the *A. nidulans* strains and quantitative RT-PCR reactions were done as previously described in [13], except that disruption of biomass for RNA isolation was prepared with a Tissue-Lyser LT (Qiagen) by treating samples for 1 min at 45 mHz. The *A. nidulans* histone 3 encoding gene, *hhtA* (AN0733) was used as an internal standard for normalization of expression levels. All primers used for quantitative RT-PCR are shown in Table 3.

Chemical characterization of mutant strains by UHPLC-DAD and LC-HRMS

All strains were grown as three point inoculations for 7 days at 37°C in the dark on solid glucose minimal, CYAs, RTO and YES media [35]. Extraction of metabolites was performed by the agar plug extraction method [38] using three 6 mm agar plugs/extract. Extracts were analyzed by UHPLC-DAD and LC-HRMS. UHPLC-DAD analysis was performed on a Dionex RSLC Ultimate 3000 (Dionex, Sunnyvale, CA) equipped with a diode-array detector. Separation was performed at 60°C on a 150 mm \times 2.1 mm ID, 2.6 μ m Kinetex C₁₈ column (Phenomenex, Torrance, CA) using a linear water/MeCN (both buffered with 50 ppm tri-fluoroacetic acid (TFA)) gradient starting from 15% MeCN to 100% over 7 min at a flow rate of 0.8 mL min⁻¹. LC-HRMS analysis was performed on a MaXis 3G QTOF (Bruker Daltonics) coupled to a Dionex Ultimate 3000 UHPLC system equipped with a 100 \times 2.0 mm, 2.6 μ m, Kinetex C-18 column. The separation column was held at a temperature of 40°C and a gradient system composed of A: 20 mM formic acid in water, and B: 20 mM formic acid in acetonitrile was used. The flow was 0.4 ml/min, 85% A graduating to 100% B in 0–10 min, 100% B 10–13 min, 85% A 13.1–15 min. For calibration, a mass spectrum of sodium formate was recorded at the beginning of each

Table 3. PCR primers used in this study.

Primer pair (fw/rv)	Forward primer	Reverse Primer	Description
JBN 2Q/3Q	GCCAAGTGTGGAATGCG	gatccccgggaattccatgCAACACTATCGCATACTCTCC	Amplifies 2 kb upstream region from <i>argB</i> (ANID_04409) for fusion PCR
JBN 4Q/5Q	aattccagctgaccaccatgCCGATCAGTAAAGCCTGTTAG	CAGGGCGTGGGAGATAGC	Amplifies 2 kb downstream region from <i>argB</i> (ANID_04409) for fusion PCR
JBN 5A/2K	catggcaattcccgggatctGGATAACCGTATTACCGCC	GGAAGAGAGGTTACACAC	Amplifies 5' <i>A. fumigatus pyrG</i> sequence including 300 bp direct repeat and native promoter for fusion PCR
JBN 4Q/2B	TGATACAGGTCTCGGTCCC	catgtgtgtcagctggaattTGCCAAGCTTAACGCGTACC	Amplifies 3' <i>A. fumigatus pyrG</i> sequence including 300 bp direct repeat and native terminator for fusion PCR
JBN 2Q/2K	GCCAAGTGTGGAATGCG	GGAAGAGAGGTTACACAC	Amplifies <i>argB</i> (ANID_04409); <i>A. fumigatus pyrG</i> upstream gene targeting fragment
JBN 4Q/5Q	TGATACAGGTCTCGGTCCC	CAGGGCGTGGGAGATAGC	Amplifies <i>argB</i> (ANID_04409); <i>A. fumigatus pyrG</i> downstream gene targeting fragment
Motni 165/185	ctgtcgauGCAGTGAGAGCGATCGCAGACACT GCATGACCATGATTACGGATTTC	cacrgauTTATTTTTTGACACCAGACCA	Amplifies the <i>E. coli lacZ</i> ORF for cloning into an <i>AsiSI/Nb.BtsI</i> USER cloning cassette. Forward primer introduces an upstream <i>AsiSI/Nb.BtsI</i> USER cloning cassette
Motni 355/354	agagcgauTAAGCTCCTCAATTGGCCC	tctcgauGCGGTAGTGATGTCTGTCTCA	Amplifies 0.5 kb of the <i>gpdA</i> promoter for cloning into an <i>AsiSI/Nb.BtsI</i>
287/288	agagcgauATGTTCTGCTCGACGCTT	tctcgauTCAGAATAAATTGCTTGGAAC	Amplifies the putative ORF of <i>A. niger</i> gene <i>fge1_pg_C_4000037</i>
289/290	agagcgauATGCGCCATCGACTCAAAA	tctcgauTTAATAGAGAGCCCATCGC	Amplifies the putative ORF of <i>A. niger</i> gene <i>e_gw1_4.316</i>
297/298	agagcgauATGAGATCTCCAGTCAA	tctcgauTCAGCATCCATGCACATGAG	Amplifies the putative ORF of <i>A. niger</i> gene <i>e_gw1_8.296</i>
299/300	agagcgauATGAGTCCGTGCTGGCCA	tctcgauTTACATATTCCATGCAAAACA	Amplifies the putative ORF of <i>A. niger</i> gene <i>e_gw1_11.945</i>
303/304	agagcgauATGGTCTACTGCGGTCCG	tctcgauCTATCCACTTAATGGTGGAC	Amplifies the putative ORF of <i>A. niger</i> gene <i>est_fge1_pg_C_150220</i>
305/306	agagcgauATGAACAGTGAACGAAAGCT	tctcgauCTATGGATTGGCCATAACCT	Amplifies the putative ORF of <i>A. niger</i> gene <i>gw1_10.247</i>
307/308	agagcgauATGGATTGAACAGAAAGTG	tctcgauCTATCTTCGAGAACCTCTT	Amplifies the putative ORF of <i>A. niger</i> gene <i>fge1_pg_C_19000192</i>
BGHA 163/502	GGTCTACTCCCTCGGTCTA	AGGGAGGCCTTGTCGGTCTT	Check primers for integration in <i>IS1</i> . Amplifies the junction between <i>A. nidulans</i> chromosome 1 and the <i>gpdA</i> promoter
BGHA 98/162	GGTTTCGTTGTCAATAAGGGAA	GTTCAAGGTGACGGTGAGAG	Check primers for integration in <i>IS1</i> . Amplifies the junction between <i>A. nidulans</i> chromosome 1 and the <i>argB</i> marker gene
JBN X64/X65	GAACGACGGAACGTGTGCTC	CTGCACAATAAGCCACGC	Detection of <i>smrA</i> by Southern blot
JBN X66/X67	ATGGTCTACTGGGTGCG	CGAGACCGATGACGACGAG	Detection of insertion into <i>IS1</i> by Southern blot
JBN X28/X29	CACCCAAACCATCTGTCGC	CTGCCAAGCCCTGCTTC	Check primers for transcription of <i>smrA</i>
JBN L39/L52	GAGGCGACGACGAAGCTG	GTGCTCTCCAGGAGTCCG	Check primers for transcription of <i>hhtA</i> (ANID_00733)

Upper case letters indicate annealing nucleotides, lower case indicate tails for user cloning.
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chromatogram using a divert valve (0.3–0.4 min). Samples were analyzed both in positive and negative ionization mode. De-replication of induced compounds were performed by comparison of accurate mass to the metabolite database Antibase2009 [39], comparison of UV spectra to published data as well as authentic standards (JH-III, Sigma Aldrich).

Chemical characterization of mutant strains by GC-MS

Volatile metabolites were collected during days 5–7 for the strains inoculated in CYAs. To collect the volatiles, a stainless steel Petri dish lid with a standard 1/4 Swagelok™ replaced the usual lid [40]. This lid possessed a standard 1/4 Swagelok fitting with PTFE insert in the centre that is used to hold a charcoal tube (SKC, 226-01). The collected volatiles were extracted from the charcoal tube with 0.3 mL of ether (Sigma Aldrich). The samples were concentrated to approximately 0.1 mL using a nitrogen flow in a GC vial and analysed using a Finnigan Focus GC coupled to a Finnigan Focus DSQ mass selective detector. The separation of the volatiles was done on a Supelco SLB™-5 MS capillary column, using He as carrier gas, at 1.2 mL/min. The injection and detection temperature was set to 220°C. One microlitre of each sample was injected into the GC–MS system. Chromatographic conditions were set to an initial temperature of 35°C for 1 min, raised at 6°C/min to 220°C and then 20°C/min to 260°C for 1 min. The separated compounds were characterized by their mass spectra generated by electron ionization (EI) at 70 eV at a scan range from m/z 35–300.

Isolation of methyl (2E,6E)-10,11-dihydroxy-3,7,11-trimethyl-2,6-dodecadienoate (JH-diol)

NID477 was cultured on 100 CYAs plates for 7 days at 37°C in the dark. The plates were homogenized using a Stomacher homogenizer and 100 mL ethyl acetate (EtOAc) +1% formic acid (FA) pr. 10 plates. The extract was filtered after 1 hour and the remaining broth was extracted with EtOAc +1% FA for 24 hours. The extract was filtered and the two fractions pooled and dried down on a freeze drier. The crude extract was separated into three phases by dissolving it in 9:1 MeOH:H₂O – Milli-Q and extracted into a heptane phase followed by a dichloromethane (DCM) phase. The DCM phase was fractionated with a 10 g ISOL Diol column, using 13 steps of stepwise Hexane-dichloromethane-EtOAc-MeOH. JH-diol was present in the DCM fraction (9.5 mg) and was purified on a Waters HPLC W600/996PDA (Milford, MA, USA) using a RP column (Phenomenex Luna C18 (2), 250×10 mm, 5 µm, Torrance, CA, USA) using a gradient of 40% MeCN (H₂O – Milli-Q (Millipore, MA, USA)) to 100% over 20 min with 50 ppm TFA and a flow of 4 mL/min. The fractions were concentrated on a rotarvap (Büchi V-855/R-215) and dried down under N₂(g) to yield 2.0 mg of JH-diol. 1 and 2D NMR characterization (1H, DQF-COSY, H2BC, HMBC and HSQC) of the compound showed that the compound was a racemic mixture with a 2:3 ratio of JH-diol a: JH-diol b. This is in agreement with an optical rotation of 0.0. The chemical shifts differed most in the reduced end of JH-diol, where the stereocenter is present, whereas the chemical shifts from C5 to C1 were overlaying. The difference of chemical shifts of the two methyl groups (H₁₂/C₁₂ and H₁₃/C₁₃) and the two CH₂ groups next to the stereocenter are due to the presence of the chiral center. The two diastereomers present in the JH-diol solution must be due to the presence of JH-diol in both the *E*- and *Z*-conformation at the C₆ and C₇ double bond. The carbon shifts are in good agreement with published data [26].

Isolation of methyl (2E,6E)-10-hydroxy-11-formyl-3,7,11-trimethyl-2,6-dodecadienoate (compound 2)

Compound 2 was present in the 60:40 DCM:EtOAc fraction (13.1 mg) of the Diol fractionation as described above and was purified on a Waters HPLC W600/996PDA (Milford, MA, USA) using a RP column (Phenomenex Luna C18(2), 250×10 mm, 5 µm, Torrance, CA, USA) using a gradient of 40% MeCN (H₂O – Milli-Q (Millipore, MA, USA)) to 100% over 20 min. with 50 ppm TFA and a flow of 4 mL/min. The collections were concentrated on a rotarvap (Büchi V-855/R-215) and dried under N₂(g) to yield 2.6 mg of compound 2.

Isolation of methyl (2E,6E)-10,11-epoxid-3,7,11-trimethyl-2,6-dodecadienoate (JH III)

JH-III was present in the 46:60 DCM:EtOAc fraction (26.2 mg) of the Diol fractionation as described for JH-diol and was purified on a Waters HPLC W600/996PDA (Milford, MA, USA) using a RP column (Phenomenex Luna C18(2), 250×10 mm, 5 µm, Torrance, CA, USA) using a gradient of 55% MeCN (H₂O – Milli-Q (Millipore, MA, USA)) to 65% over 20 min. with 50 ppm TFA and a flow of 4 mL/min. The fractions were concentrated on a rotarvap (Büchi V-855/R-215) and dried under N₂(g) to yield 1.4 mg of JH-III. However, the purified JH-III degraded before NMR experiments could be conducted. Instead, JH-III was identified based on comparison of accurate mass and retention time with authentic standard. HRMS (m/z): [M+H]⁺ calcd. for C₁₆H₂₇O₃, 267.1955; found, 267.1957; [M+Na]⁺ calcd. For C₁₆H₂₆O₃Na, 289.1780; found, 289.1774.

NMR studies and structure elucidation

NMR spectra were acquired in DMSO-*d*₆ on a Varian Unity Inova 500 MHz spectrometer for JH-diol and JH-III and on a Bruker Avance 800 MHz spectrometer at the Danish Instrument Center for NMR Spectroscopy of Biological Macromolecules for compound 2 using standard pulse sequences. The spectra were referenced to this solvent with resonances δ_H = 2.49 and δ_C = 39.5.

Characterization data of methyl (2E,6E)-10,11-dihydroxy-3,7,11-trimethyl-2,6-dodecadienoate (JH-diol)

NMR data for JH-diol-a: ¹H NMR (500 MHz, DMSO-*d*₆): δ 5.65 (s, 1 H), 5.07 (m, 1 H), 4.25 (d, J = 5.6 Hz, 1 H), 4.01 (s, 1 H), 3.57 (s, 3 H), 3.02 (ddd, J = 10.0, 5.6, 2.5 Hz, 1 H), 2.15 (m, 2 H), 2.15–2.11 (m, 2 H), 2.09 (s, 3 H), 1.87 (m, 2 H), 1.60 (m, 1 H), 1.56 (s, 3 H), 1.15 (m, 1 H), 1.02 (s, 3 H), 0.97 (s, 3 H); ¹³C NMR (125 MHz): δ 166.1, 159.7, 135.9, 122.2, 114.7, 76.6, 71.5, 50.4, 39.7, 36.2, 29.4, 26.1, 25.1, 24.2, 18.2, 15.7.

NMR data for JH-diol-b: ¹H NMR (500 MHz, DMSO-*d*₆): δ 5.65 (s, 1 H), 5.08 (m, 1 H), 3.74 (dd, J = 10.0, 3.0 Hz, 1 H), 3.57 (s, 3 H), 2.15 (m, 2 H), 2.15–2.11 (m, 2 H), 2.10 (m, 1 H), 2.09 (s, 3 H), 1.96 (m, 1 H), 1.57 (s, 3 H), 1.48 (m, 1 H), 1.41 (m, 1 H), 1.21 (s, 3 H), 1.08 (s, 3 H); ¹³C NMR (125 MHz): 166.1, 159.7, 134.9, 123.0, 114.7, 82.6, 79.4, 50.4, 39.7, 35.7, 29.2, 27.7, 25.1, 22.8, 18.2, 15.6. HRMS (m/z): [M+H]⁺ calcd. For C₁₆H₂₈O₄, 285.2060; found, 285.2025; [M+Na]⁺ calcd. For C₁₆H₂₈O₄Na, 307.1885; found, 307.1887; [α]_D = 0.0 (MeOH).

Characterization data of methyl (2E,6E)-10-hydroxy-11-formyl-3,7,11-trimethyl-2,6-dodecadienoate (compound 2)

¹H NMR (800 MHz, DMSO-*d*₆): δ 8.23 (s, 1 H), 5.65 (q, J = 1.0 Hz, 1H), 5.05 (t, J = 6.9, 1 H), 4.62 (s, 1 H), 4.52 (d, J = 10.2 Hz, 1 H), 3.57 (s, 3 H), 2.15 (m, 2 H), 2.11 (m, 2 H), 2.09 (d, J = 1 Hz, 3 H), 1.93 (m, 1 H), 1.84 (m, 1 H), 1.75 (m, 1 H),

1.55 (s, 3 H), 1.46 (m, 1 H), 1.04 (s, 3 H), 1.03 (s, 3 H); ^{13}C NMR (200 MHz): δ 166.2, 162.3, 159.9, 134.6, 123.4, 114.7, 79.7, 70.2, 50.4, 39.6, 35.7, 26.9, 25.2, 25.1, 25.1, 18.3, 15.6; HRMS (m/z): $[\text{M}+\text{H}]^+$ calcd. for $\text{C}_{17}\text{H}_{29}\text{O}_5$, 313.2010; found, 313.2010. $[\text{M}+\text{Na}]^+$ calcd. for $\text{C}_{17}\text{H}_{28}\text{O}_5\text{Na}$, 335.1828; found, 335.1831.

Characterization data of methyl (2, 6, 10) -3,7,11-trimethyl-2,6-dodecadienoate (MF)

HRMS (m/z): $[\text{M}+\text{H}]^+$ calcd. for $\text{C}_{16}\text{H}_{27}\text{O}_2$, 251.2006; found, 251.2007.

Confrontation with *D. melanogaster* larvae

Fungal strains were point-inoculated (1000 conidia in 1 μl Ringer solution) on 3 ml standard *Drosophila* medium [30] filled in 3.5 cm diameter Petri dishes. Prior to the transfer of ten sterile *D. melanogaster* larvae per plate, colonies were pre-incubated for two days at 25°C and constant darkness. Colonies were exposed to insects for four days. Subsequently, insects were removed and the plates snap frozen for metabolite profile analysis. Quantitative metabolite profile analyses were performed on groups of five biological replicates. Statistical analysis was performed with pairwise comparisons using the student's t-test procedure. Evaluation of insect fitness followed the procedure described in Trienens et al. [30]. We confronted the larval stage of the fruit fly *Drosophila melanogaster* (wild type Oregon R strain) with the reference NID545 or the JH-producer strain NID477. Sterile two-day first-instar larvae were exposed to *A. nidulans* colonies growing on autoclaved

standard *Drosophila* culture medium in 2 ml micro-tubes. There were $N = 20$ experimental units per fungal treatment and $N = 10$ mold-free control units. Insect developmental success was monitored in terms of (1) larva-to-pupa survival and development time, (2) emergence of flies, and (3) fly dry weight. Short development time and high body mass are considered to be positively correlated with fitness in *Drosophila* [41]. Experimental tubes were checked for pupae and emerged flies at about 2 p.m. each day for a total of 14 days after larval transfer. Emerged flies were removed from the tubes and stored deep-frozen. Subsequently, flies were lyophilized for 24 hours and the dry weight of all flies within each experimental unit was determined as a single value using a micro-balance.

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Author Contributions

Conceived and designed the experiments: MTN MLK MR CHG MRA BGH UM TOL. Performed the experiments: MTN MLK MR CHG. Analyzed the data: MTN MLK MR CHG MRA BGH UM TOL. Contributed reagents/materials/analysis tools: MTN MRA MR BGH CHG. Wrote the paper: MTN. Reviewed the manuscript: MLK MR DCA JBN CHG MRA BGH UHM TOL.

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2.2 Heterologous production of fungal secondary metabolites in Aspergilli

This section comprises a review “Heterologous production of fungal secondary metabolites in Aspergilli” submitted to *Frontiers in Microbiology*. This covers the strategies used for heterologous expression of biosynthetic pathways in aspergilli until October 2014.

Heterologous production of fungal secondary metabolites in *Aspergilli*

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Abstract

Fungal natural products comprise a wide range of compounds. Some are medically attractive as drugs and drug leads, some are used as food additives, while others are harmful mycotoxins. In recent years the genome sequence of several fungi has become available providing genetic information of a large number of putative biosynthetic pathways. However, compound discovery is difficult as the genes required for the production of the compounds often are silent or barely expressed under laboratory conditions. Furthermore, the lack of available tools for genetic manipulation of most fungal species hinders pathway discovery. Heterologous expression of the biosynthetic pathway in model systems or cell factories facilitates product discovery, elucidation and production. This review summarizes the recent strategies for heterologous expression of fungal biosynthetic pathways in *Aspergilli*.

Introduction

Filamentous fungi produce a plethora of secondary metabolites, SMs, like polyketides, terpenes, and non-ribosomal peptides. Several fungal SMs dramatically impact human life either because they are harmful mycotoxins, like carcinogenic aflatoxin (Eaton and Gallagher, 1994) and fumonisin (Voss and Riley, 2013), or because they are used to efficiently combat human disease e.g. penicillin and lovastatin (Campbell and Vederas, 2010). Importantly, analyses of fully sequenced fungi show that the number of SMs known to be produced by these fungi is too low to account for the number of genes and gene clusters that potentially may lead to production of SMs (Szewczyk et al., 2008). This strongly suggests that the chemical diversity of the metabolomes produced by filamentous fungi is much larger than what is currently known, and it is therefore very likely that new harmful mycotoxins and new blockbuster drugs await discovery.

The rapid accumulation of fully sequenced genomes has accelerated the discovery of novel SMs dramatically. However, this sequence resource cannot be directly translated into chemical structures of new compounds despite that genes and gene clusters are often readily identified by bioinformatics tools (Khaldi et al., 2010; Blin et al., 2013; Andersen et al., 2013). For example, the exact structures of products released by fungal type I polyketide synthases are difficult to predict due to the iterative use of the different catalytic domains in these enzymes. Similarly, subsequent decorations performed by tailoring enzymes encoded by other genes in the cluster towards formation of the mature end product(s) are complex and not easy to predict. Another challenge is that many SMs are not readily

produced under laboratory conditions although several approaches have been successfully employed to activate silent clusters (for reviews, see (Brakhage and Schroeckh, 2011; Chiang et al., 2011; Klejnstrup et al., 2012; Wiemann and Keller, 2014; Yaegashi et al., 2014). To link novel SMs to genes, and to map novel biosynthetic pathways, extensive genetic manipulations of the strains are typically required. Since, most new gene clusters uncovered by sequencing projects will be situated in fungi with no available genetic tools, this type of analysis may not be straight forward. Moreover, it may be difficult to purify sufficient amounts of a desired compound from these fungi to allow for thorough characterization of its bioactivity. An alternative approach is to transfer genes and gene clusters to hosts with strong genetic toolboxes thereby facilitating product discovery, production and characterization. This review will focus on recent strategies for heterologous expression of SM pathways in Aspergilli based expression platforms. We will mainly describe examples aiming at PK production, but similar strategies can be used for production of all types of fungal SMs.

Host choice for heterologous expression of fungal secondary metabolites

Heterologous expression of SM genes has mainly been performed in baker's yeast *Saccharomyces cerevisiae* (Tsunematsu et al., 2013) and in the filamentous fungi *Aspergillus oryzae* and *Aspergillus nidulans*. Each of these model organisms offers specific advantages. For *S. cerevisiae* a superior genetic toolbox for strain construction has been developed and novel genes can easily be engineered into a wealth of single- and multi-copy expression plasmids or into chromosomes. For example, gene targeting and fusion of DNA fragments by homologous recombination (HR) is highly efficient in *S. cerevisiae*. Moreover, *S. cerevisiae* contains an insignificant endogenous secondary metabolism (Siddiqui et al., 2012). This fact simplifies the analysis of strains equipped with new pathways as they are not complicated by the presence of a multitude of other SMs; and the risk of undesirable side reactions due to cross chemistry between the novel and endogenous pathways is minimized. However, lack of secondary metabolism also means that yeast is not naturally geared for SM production and may contain limiting amounts of, or even lack, relevant building blocks (Kealey et al., 1998; Mutka et al., 2006). Moreover, localization of relevant enzymes for aflatoxin production into specialized vesicles in *A. parasiticus* indicate that fungi may possess specialized compartments for SM production, which yeast may not contain (Roze et al., 2011); and as introns are few in *S. cerevisiae* (Spingola et al., 1999) and differ from those in filamentous fungi (Kupfer et al., 2004), mRNA splicing could be problematic. For these reasons filamentous fungi may often be more appropriate for heterologous SM production. *A. oryzae* is often used for this purpose because it possesses a limited endogenous secondary metabolism and *A. nidulans* because a strong genetic toolbox has been developed for this fungus (for review see, (Meyer, 2008; Meyer et al., 2011)). Importantly, the recent development of efficient tools for gene targeting in filamentous fungi, including strains where random integration is minimized due to mutation of genes required for non-homologous end-joining (Ninomiya et al., 2004; Takahashi et al., 2006; Nayak et al., 2006), has further stimulated the use of these organisms as hosts for SM pathway reconstitution experiments.

Heterologous expression of polyketide synthases

The fact that the product(s) released by fungal type I PKS synthases cannot easily be predicted from their primary sequence has sparked a major interest in expressing PKS genes in model fungi with the aim of identifying these products. In yeast, two 2 μ based multi-copy plasmids harboring the 6-methylsalicylic acid (6-MSA) synthase gene from *Penicillium patulum* and the PKS activating

PPTase gene from *Bacillus subtilis* were successfully used to produce 6-MSA (Kealey et al., 1998). Similarly production of green pigment has been achieved in a *wAA yAA* (white) *A. nidulans* (Holm, 2013) via co-expression of the PKS gene *wA* and laccase gene *yA* harbored on two AMA1 (Aleksenko and Clutterbuck, 1997) based plasmid. However, if multiple plasmids are needed to form a complex end-product, these vectors may have limited value since sufficient markers may not be available, and since 2 μ and AMA1 plasmids segregate unevenly during mitosis (Albertsen et al., 2011; Holm, 2013; Jensen et al., 2014).

More stable expression has been achieved by integrating PKS genes randomly into the genome of a model filamentous fungus via the non-homologous end-joining pathway. Using this concept, Fujii et al., successfully linked 6-MSA production to the PKS gene *atX* from *A. terreus* by expressing *atX* host *A. nidulans* (Fujii et al., 1996). Considering that foreign SMs may be toxic in the new host, it is advisable to employ an expression strategy that minimizes this risk. For production of 6-MSA and enniatins in *A. nidulans* and *A. niger*, this was achieved by fusing the PKS and NRPS genes to the inducible promoters, *amyB* (Fujii et al., 1996) and Tet-on (Richter et al., 2014), respectively. Over the years, a number of other PKS genes have been linked to products using this strategy in *A. nidulans* and *A. oryzae* including the PKS genes for production of 1,3,6,8-tetrahydroxynaphthalene, alternapyrone and 3-methylorcinolaldehyde by (Fujii et al., 1999, 2005; Bailey et al., 2007).

Random integration may trigger unpredictable pleiotropic effects that alter the expression of neighboring genes, hence, complicating subsequent analyses (Verdoes et al., 1995; Palmer and Keller, 2010). Moreover, since multiple copies of the gene often integrate simultaneously into the same site, strains may suffer genetic instability and lose expression over time. Taking advantage of the development of strains and techniques for efficient gene targeting, these problems can be eliminated by inserting genes into a defined locus. This facilitates not only subsequent strain characterization, but also sets the stage for experiments analyzing mutant varieties of the gene where equal expression levels of the alleles are important to fairly judge the impact of individual mutations. Using this approach, Hansen et al. demonstrated that *mpaC* from *Penicillium brevicompactum* encodes a PKS producing 5-methylorsellinic acid (Hansen et al., 2011). In this case, *mpaC* was introduced into a defined site, *ISI*, on chromosome I of *A. nidulans*, which supports expression of non-toxic genes in a variety of tissues without affecting fitness. Moreover, to simplify the integration of genes into *ISI*, a set of vectors pre-equipped with targeting sequences, genetic markers, promoters and terminators and a USER-cloning cassette (Nour-Eldin et al., 2006) allowing for seamless ligation free insertion of relevant genes into the vector was developed. Using this technology, *ausA*, from *A. nidulans*, and *yanA*, from *A. niger*, have been shown to encode PKSs producing 3-,5-dimethyl orsellinic acid and 6-MSA, respectively (Nielsen et al., 2011; Holm et al., 2014). In a variation of this approach, Chiang et al. used fusion PCR to merge an *alcA* promoter and PKS genes followed by integration into the *wA* locus of *A. nidulans*. Correctly targeted transformants could therefore easily be identified as white colonies. The authors expressed nine non-reducing (NR) PKS genes from *A. terreus* in this manner and identified six products. Heterologous production of PKs is complicated by the fact that not all synthases possess a domain providing a product release mechanism (Du and Lou, 2010; Awakawa et al., 2009) and by the fact that some PKSs require a starter unit different from Ac-CoA (Hoffmeister and Keller, 2007). In the study by Chiang et al., two of the nine NR-PKSs analyzed did not contain such a domain and for one, a product was achieved by co-expressing a gene encoding a thioesterase activity. In addition, two NR-PKS were predicted to employ unusual starter

units. For one NR-PKS, production of this starter unit was successfully delivered by co-expressing a gene encoding a highly reducing PKS and the collaborative effort of the two enzymes resulted in production of an intermediate for production of asperfuranone (Chiang et al., 2013).

Transfer of gene clusters to heterologous hosts

Reconstitution of most SM pathways depends on the expression of multiple genes since the SM scaffold delivered by the synthase is further decorated by tailoring enzymes. Moreover, genes providing transcription factors, transporters and/or a resistance mechanism may also be required. Construction of strains for heterologous end-product production is therefore a major challenge as it requires not only transfer, but also activation, of large gene clusters. Two principles are generally employed for constructing DNA fragments that allow transfer of gene clusters into another fungal host. Firstly, DNA fragments harboring entire, or a large part of, gene clusters have been identified in cosmid/fosmid libraries and transferred into vectors with a selectable fungal marker (Figure 1(A)). Secondly, PCR fragments covering the gene cluster have been stitched together using a variety of methods including USER Fusion, Gateway cloning and yeast recombination to create suitable transformation vectors (Figure 1(B)). When gene clusters have been transformed into the host, activation has been achieved by three different methods. Firstly, in cases where the native gene cluster harbors a TF gene, it has been possible to activate the genes in the cluster by equipping the TF gene with a constitutive or inducible promoter known to work in the host. Secondly, in gene clusters without a TF gene, activation has been achieved either by overexpressing the global regulator LaeA or by individually swapping cluster gene promoters for constitutive or inducible promoters. Like for integration of PKS genes, and for the same reasons, integration strategies based on random or directed integration have been used (Figure 1(C)). In many cases these strategies have been combined and successful examples are provided below.

Cosmids harboring the entire penicillin biosynthetic pathway from *P. chrysogenum* were introduced to *Neurospora crassa* and *A. niger*, resulting in the production of penicillin (Smith et al., 1990). Similarly, cosmids harboring the citrinin biosynthetic pathway from *Monascus purpureus* and the monacolin K gene cluster from *Monascus pilosus* were individually integrated into random positions in the genome of *A. oryzae*. In the case of citrinin, the transformant directly produced citrinin, but in small amounts. However, as the cluster contains a TF gene, additional copies of the activator gene (*ctnA*) controlled by the *A. nidulans trpC* promoter were subsequently introduced in the strain to boost production. Impressively, this resulted in a 400 fold increase of citrinin production (Sakai et al., 2008). In the case of monacolin K, the gene cluster does not contain a TF gene. However, by overexpressing a gene encoding the global activator LaeA, the cluster was successfully activated as the strain produced monacolin K (Sakai et al., 2012). A limitation of this strategy may be difficulties in isolating cosmids containing a fragment that harbors the entire gene cluster, especially if clusters are large. For example, the reconstruction of the terrequinone A gene cluster in *A. oryzae* was based on a fosmid containing an incomplete gene cluster. The remaining part of the cluster was subsequently obtained by PCR, cloned into a vector and transformed into the *A. oryzae* strain harboring the partial terrequinone A gene cluster (Sakai et al., 2012).

Several PCR based strategies have been used for transferring gene clusters from the natural producer

to a model fungus. For clusters harboring a TF gene, PCR fragments covering the entire gene cluster have been amplified, fused and inserted via a single cloning step into vectors predestined for site specific integration in the genome of the host by HR. Multiple PCR fragments can be orderly assembled by different strategies. For example, PCR fragments of the geodin and neosartoricin B clusters were physically linked by *E. coli* based USER fusion and by yeast based HR, respectively (Nielsen et al., 2013; Yin et al., 2013). Importantly, in both cases the promoter controlling expression of the TF gene was swapped for a strong constitutive promoter during the cluster re-assembly process. Large inserts (> 15 kb) may not be propagated stably in a cloning vector and large clusters need to be subdivided into smaller fragment cassettes, which together represent the entire cluster. Multiple subsequent integrations depend on marker recycling, which can be achieved by using *pyrG* as a selectable/counterselectable marker. A faster method employs a two marker system for cluster transfer (Nielsen et al., 2013). During one transformation cycle, one of the markers is used to select for integration of the first cluster cassette and the other marker for the next cassette. By ensuring that integration of one cassette eliminates the marker contained by the preceding cassette, numerous cluster cassettes can be integrated sequentially by alternating the use of the two markers. Advantageously, when the gene clusters is inserted in a controlled manner it can be subjected to further genetic dissection to clarify the biochemical pathway towards end product. With the geodin cluster this was exploited to demonstrate that *gedL* encodes a halogenase using sulochrin as substrate (Nielsen et al., 2013).

PCR based reconstruction of clusters that do not contain an activating TF gene requires more elaborate genetic engineering as all cluster genes need to be equipped with new promoters and terminators. In one strategy, cluster ORFs were inserted either individually or in pairs into expression cassettes in plasmids carrying different selection markers. Using this approach several small gene clusters containing four to five genes have been, fully or partially, reconstituted by randomly introducing the genes into the genome of *A. oryzae*. Several SMs have been achieved by this method including tennelin, pyripyropene, aphidicolin, terretonin, and andrastin A (Heneghan et al., 2010; Itoh et al., 2010; Fujii et al., 2011; Matsuda et al., 2012, 2013). Construction of larger clusters in *A. oryzae* has been limited by the number of available markers. To bypass this problem, Tagami et al. used the high co-transformation frequency with *A. oryzae* to integrate two vectors in one round of transformation using selection for only one marker. This allowed for reconstituting clusters with six and seven genes for production of paxilline and aflatoxin, respectively (Tagami et al., 2013, 2014). Addressing the same problem, Gateway cloning was used to construct expression vectors containing up to four genes (Pahirulzaman et al., 2012; Lazarus et al., 2014). Utilizing this approach Wasil et al. expressed different combinations of the synthase and tailoring genes from the aspyridone pathway from *A. nidulans* in *A. oryzae* (Wasil et al., 2013). An alternative approach to save markers is to generate synthetic polycistronic genes where all genes in the construct are under the control of a single promoter and where all ORFs are separated by a sequence encoding the viral 2A peptide that results in co-translational cleavage, hence, resulting in the formation of independent enzymes (Kim et al., 2011). Using this concept Unkles et al. reconstituted the penicillin gene cluster from *P. chrysogenum* as a single three ORF polycistronic gene by yeast mediated HR. Random genomic integration of this construct resulted in penicillin production in *A. nidulans* (Unkles et al., 2014).

A strategy for gene cluster activation based on promoter/terminator swapping has also been implemented in gene cluster transfer methods where genes are inserted into defined integration sites

(Mikkelsen et al., 2012; Hansen et al., 2012; Chiang et al., 2013). Specifically, expression plasmids containing one to two cluster genes were constructed by USER cloning or by fusion PCR and integrated into the expression sites in *S. cerevisiae* and *A. nidulans* to allow for production of the pigment precursor rubrofusarin in yeast (Rugbjerg et al., 2013) and for partial and fully reconstitution of the pathways for mycophenolic acid and asperfuranone production, respectively, in *A. nidulans* (Hansen et al., 2012; Chiang et al., 2013).

Perspectives

The rapid development of molecular tools for cluster transfer and re-engineering in heterologous hosts is now at a stage where high-throughput experiments can be performed, and we therefore predict that novel SMs, genes, pathways and enzymes routinely will be discovered using this approach. For now most efforts have been proof of principle cases analyzing genes and gene clusters from genetically well-characterized organisms, but the next wave of breakthroughs will likely concern SMs originating from genetically exotic fungi. In addition, the natural reservoir of SMs will likely expand dramatically as synthetic biology based approaches using bio-bricks of promoters, terminators and SM genes are combined in intelligent or in random ways in model fungi to deliver compounds that nature never invented. Together, we envision that heterologous production will serve as a major driver for SM discovery and development delivering compounds that can be used in the food and -pharma industries. Accordingly, physiologically well-characterized fungal cell factories should preferentially be employed as platforms for novel SMs discovery and development. These fungi display superior fermentation properties and extensive metabolic engineering toolboxes, hence, shortening the way towards large scale production.

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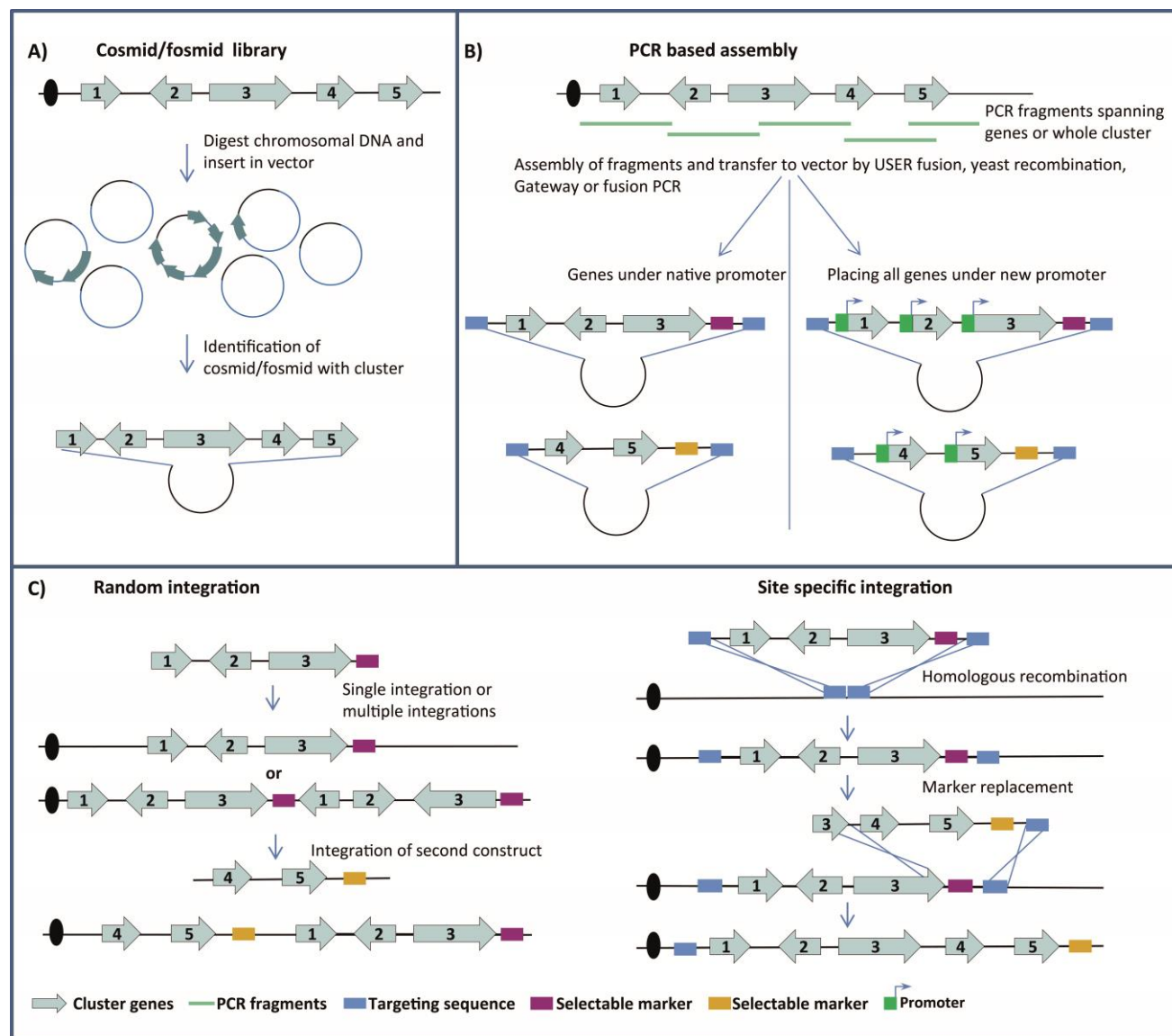
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Figure legends

Figure 1. Overview of principles employed for constructing DNA fragments (A) & (B) and for integration in host genomes (C).



2.3 Heterologous reconstitution of the intact geodin gene cluster in *Aspergillus nidulans* through a simple and versatile PCR based approach

This section contains the paper “Heterologous reconstitution of the intact geodin gene cluster in *Aspergillus nidulans* through a simple and versatile PCR based approach.” In this study we introduce a simple PCR based approach for the transfer of gene clusters.

Heterologous Reconstitution of the Intact Geodin Gene Cluster in *Aspergillus nidulans* through a Simple and Versatile PCR Based Approach

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Abstract

Fungal natural products are a rich resource for bioactive molecules. To fully exploit this potential it is necessary to link genes to metabolites. Genetic information for numerous putative biosynthetic pathways has become available in recent years through genome sequencing. However, the lack of solid methodology for genetic manipulation of most species severely hampers pathway characterization. Here we present a simple PCR based approach for heterologous reconstitution of intact gene clusters. Specifically, the putative gene cluster responsible for geodin production from *Aspergillus terreus* was transferred in a two step procedure to an expression platform in *A. nidulans*. The individual cluster fragments were generated by PCR and assembled via efficient USER fusion prior to transformation and integration via re-iterative gene targeting. A total of 13 open reading frames contained in 25 kb of DNA were successfully transferred between the two species enabling geodin synthesis in *A. nidulans*. Subsequently, functions of three genes in the cluster were validated by genetic and chemical analyses. Specifically, ATEG_08451 (*gedC*) encodes a polyketide synthase, ATEG_08453 (*gedR*) encodes a transcription factor responsible for activation of the geodin gene cluster and ATEG_08460 (*gedL*) encodes a halogenase that catalyzes conversion of sulochrin to dihydrogeodin. We expect that our approach for transferring intact biosynthetic pathways to a fungus with a well developed genetic toolbox will be instrumental in characterizing the many exciting pathways for secondary metabolite production that are currently being uncovered by the fungal genome sequencing projects.

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Introduction

Fungal natural products constitute a rich resource for bioactive secondary metabolites [1,2]. To fully exploit this potential, it is essential to identify the genes required for the biosynthesis of these compounds. This process is becoming progressively easier due to the rapidly increasing number of fungal genomes that have been fully sequenced; and since the genes involved in the production of a given secondary metabolite often cluster together in the same region of a chromosome [1–3]. Importantly, the genome sequencing projects have revealed that the number of putative gene clusters for secondary metabolite production greatly exceeds the number of known natural products in a given fungus, hence, indicating that most fungal compounds are yet to be discovered.

The prerequisite for genetic exploration of the huge reservoir of undiscovered biosynthetic pathways is solid methodologies for cultivating, propagating and genetically manipulating the producing species. However, the vast majority of newly sequenced organisms fail to meet these requirements, hence, hampering

pathway elucidation and exploitation. An attractive solution to this problem is to transfer pathways into another fungus where the methodology is well established. This approach has been used successfully to investigate several gene clusters [4–6]. All of these studies apply a strategy where individual genes in a cluster are PCR amplified, cloned, and integrated sequentially into either a random or defined locus. One advantage of this strategy is that it allows easy engineering of individual genes prior to integration in the host strain. In addition, it is possible to insert the foreign gene(s) into a well characterized locus that supports high expression levels [7]. Inserting the genes into a known locus also simplifies strain validation. In a recent example of this strategy, Itoh et al. [6] transferred five genes from *Aspergillus fumigatus* to *A. oryzae* allowing the authors to deduce the biosynthetic route for the meroterpenoid pyropyripene A. However, the strategy may be limited to reconstitution of simple pathways depending on only a small number of genes since assembly of multistep pathways will require several rounds of tedious iterative integration steps or enough genetic markers.

For multistep pathways it is therefore desirable to transfer entire gene clusters from the natural producer to the expression host in one or a few steps. This requires a host, which can efficiently express all genes in the cluster and correctly splice the resulting transcripts. Assembly of multiple genes and PCR fragments can efficiently be performed in *Saccharomyces cerevisiae* by recombination based methods [8]. However, the demand for splicing disfavors *S. cerevisiae* as expression host for this gene transfer strategy as only little splicing occurs in yeast. In contrast, correct and efficient splicing of heterologous transcripts by another filamentous fungus is likely. Activation of the cluster in the new host requires as a minimum that the chromatin structure at the insertion site is in an open configuration and that transcription factors exist that recognize the individual promoters in the cluster. The latter may be facilitated by the fact that many clusters appear to contain one or more genes that encode transcription factors. In a pioneering study, Bergmann et al. showed that expression of such a gene activated the entire aspyridone gene cluster in *A. nidulans* [9]. The potential of transferring entire clusters to an expression host has been demonstrated by Sakai et al. who managed to produce citrinin in *A. oryzae* [10]. To obtain this feat, they isolated and transformed a cosmid from a *Monascus purpureus* library containing all six genes required for production of citrinin into this production host. Here constitutive expression of the citrinin pathway regulator encoded by *ctnA* dramatically increased citrinin production in the heterologous host. However, construction and screening of cosmid libraries is not simple and a versatile PCR based method that facilitates the transfer of entire gene clusters from the natural producer to the expression host is desirable.

We have previously developed a versatile PCR based expression platform that can be used for heterologous expression in *A. nidulans* of one or a pair of genes from the defined locus *ISI*, which supports a high level of gene expression [4,7]. Here, we demonstrate how this platform can be expanded to allow transfer of an entire gene cluster from another fungus into *ISI*.

Results and Discussion

2.1 Method for PCR based reconstruction of fungal gene clusters in a heterologous host

Our method for transfer of large DNA fragments relies on successive gene targeting events that introduce ~15 kb fragments into a defined locus. In the example of the method presented here, we transfer a gene cluster into *ISI* taking advantage of a vector set we have previously developed for this purpose [7]. In our method, fragments covering the entire gene cluster are PCR amplified, combined via USER fusion into ~15 kb fragments, and inserted into the integration vector by USER cloning (Figure 1A) [11,12]. The first fragment to be integrated is assembled in the vector and integrated into *ISI* as we have described previously for integration of single genes [7]. The following fragments are integrated as an extension of the previous one by using one of two different markers, *argB* and *pyrG*, for selection. In principle, an indefinite number of integrations can be done, since the marker from the previous integration is excised as the new fragment with the other marker integrates (Figure 1B). This principle is referred to as re-iterative gene targeting [13]. Importantly, marker replacements allows for a simple selection scheme for identification of correctly targeted strains. If, as in our case, the *pyrG* marker is flanked by a direct repeat, we recommend to use the *pyrG* marker in the last integration step, as the *pyrG* marker subsequently can be removed by direct repeat recombination if desirable [14]. In this manner both markers are available in the finalized strain providing a marker repertoire for additional genetic engineering.

In the present study we demonstrate the potential of our method by transferring the geodin gene cluster from *A. terreus* to *A. nidulans*. This cluster was chosen, firstly, because it contains a gene encoding a putative transcription factor, which potentially could facilitate activation of the other genes in the cluster. Secondly, the biosynthetic pathway for geodin production is partially characterized (Figure 2) [15–21], which simplifies the delineation of the cluster size. Thirdly, the geodin pathway shares several steps with the monodictyphenone pathway including production of several common intermediates/products e.g. emodin [22]. We therefore envisioned, that the chance of producing geodin in *A. nidulans* would be increased, as the corresponding endogenous enzymes could complement geodin enzymes that might not be functional. Moreover, shared intermediates would likely be non-toxic to the host.

2.2 Delineation of the putative geodin producing gene cluster in *A. terreus*

Three enzymes involved in geodin production have previously been linked to genes. Specifically, dihydrogeodin oxidase, a polyketide synthase, and a thioesterase are encoded by ATEG_08458 [18], ATEG_08451 [19–21], and ATEG_08450 [20], respectively, see *Aspergillus* Comparative Sequencing Project database (Broad Institute of Harvard and MIT, <http://www.broadinstitute.org/>). As indicated by the gene numbers these genes localize in close proximity to each other strongly suggesting that a gene cluster responsible for production of geodin exists. Three additional enzymes required for geodin biosynthesis have been characterized biochemically *in vitro*: emodin anthrone oxygenase [15], emodin O-methyltransferase [16] and questin oxygenase [17]. Moreover, the occurrence of chlorine atoms in geodin suggests the involvement of a halogenase.

To explore the possibility that genes encoding these four enzymatic activities were also present in this region, we examined all annotated open reading frames (ORFs) positioned between ATEG_08458 and ATEG_08450, as well as 20 kb upstream of ATEG_08458 and downstream of ATEG_08450. Among the ORFs in this region, none had a functional annotation corresponding to these enzymatic activities. We therefore subjected all annotated ORFs in this regions to a functional prediction using the BLAST algorithm [23] from NCBI and the HHpred software [24]. This analysis uncovered three genes that could encode putative methyltransferases (ATEG_08449, ATEG_08452 and ATEG_08456), one ORF that may encode an oxygenase carrying out a Baeyer-Villiger oxidation (ATEG_08459), and a putative halogenase (ATEG_08460), see Table 1.

Unexpectedly, none of the annotated ORFs were found to encode the emodin anthrone oxygenase (Figure 2). To investigate this apparent dilemma, we searched the literature for other oxygenases catalyzing a similar reaction. Via this effort, we found an oxygenase that catalyzes conversion of norsolorinic acid anthrone to norsolorinic acid, a step towards aflatoxin production in *A. flavus* [25]. This recently identified enzyme is encoded by the gene *hypC*. Inspired by these findings, we used the sequence of HypC to conduct pair-wise alignments to putative proteins encoded by alternative ORFs in the proposed geodin gene cluster. One short putative ORF encodes a protein of 150 amino acid residues with an overall identity of 34% with the 210 residues of HypC. Moreover, the conserved amino acid residues were primarily positioned in catalytic regions or conserved domains (Figure S1, [25]). Interestingly, the putative ORF is oriented in the opposite direction of ATEG_08457. This strongly indicates that the region at ATEG_08457 is wrongly annotated and contains two separate ORFs that we now denote ATEG_08457-1 (the originally

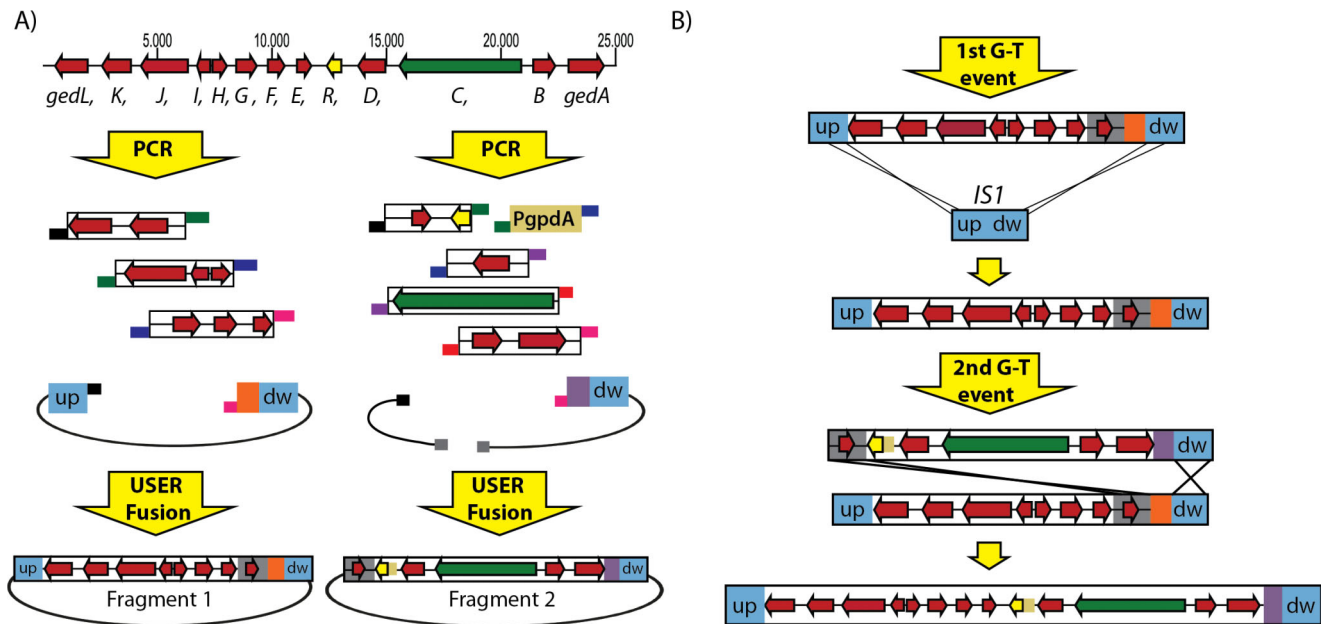


Figure 1. Schematic overview of the PCR based USER cloning strategy for transfer of entire gene clusters from one fungus to another. In the illustrated case, the geodin gene cluster in *A. terreus* is PCR amplified, cloned, and integrated into the *IS1* locus in *A. nidulans*. A) ORFs *GedA-GedL* are depicted as arrows. The yellow and green arrows represent the ORFs encoding the transcription factor and the PKS, respectively. Remaining ORFs are represented by red arrows. Arrow size is proportional to ORF length and arrow direction indicates genomic orientation. Numbers above the gene cluster specify sequence in base pairs. Genomic DNA fragments and cloning vectors are amplified as PCR products using primers extended with uracil-containing tails. The tails contain matching sequences (indicated by identical colors) allowing for PCR product assembly in a single USER Fusion reaction. For the geodin cluster, all putative ORFs are fused into two fragments, which are individually inserted into a vector prepared for gene targeting. Blue boxes labeled up (upstream) and dw (downstream) represent targeting sequences for homologous recombination into *IS1* in the first gene-targeting event. The targeting sequences in the second integration event are represented in gray and blue and consist of the overlapping region between Fragment 1 and 2 and the downstream part of *IS1*, respectively. Genetic markers used for selection are depicted in orange (*argB*) and purple (*AFpyrG*). The sizes of uracil-containing tails, vector elements and *PgpdA* fragment are not drawn to scale. B) The first gene-targeting event introduces the first fragment into *IS1* by homologous recombination between *IS1* up and down-sequences as indicated. The second gene-targeting event introduces the second fragment using the overlapping region of the Fragment 1 and 2 (gray) and the downstream section of *IS1* as targeting sequences. Note that additional DNA can be inserted in subsequent gene-targeting events. For example, a third fragment can be inserted by using the downstream end of fragment 2 and the downstream region of *IS1* as targeting sequences. See text for details concerning use and recycling of markers.

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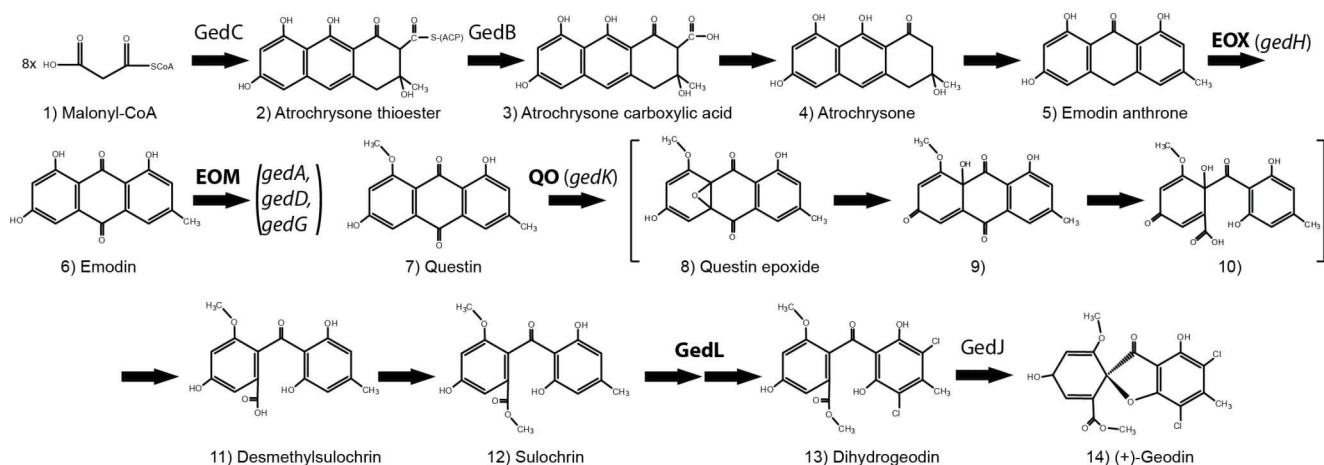


Figure 2. Proposed pathway for geodin production. The PKS (ACTS, [20]), thioesterase (ACTE, [20]) and dihydrogeodin oxidase previously linked to genes as well as the sulochrin halogenase identified in this study (highlighted in bold) are denoted by their *ged*-annotation. Enzymatic reactions for which the enzyme has been characterized but the gene not identified are marked in bold as EOX = emodin anthrone oxygenase, EOM = emodin-O-methyltransferase and QO = questin oxygenase. Reactions involving compounds 8-10 shown in brackets are inferred reactions proposed by Henry and Townsend based on a similar intra-molecular rearrangement in aflatoxin biosynthesis [26,27].

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Table 1. Summary of characterized and putative ORFs in the *A. terreus* geodin gene cluster.

BROAD annotation	Proposed annotation	Enzyme class	Validation	Reference
ATEG_08449	GedA	Putative O-methyl transferase*	BLAST, Hhpred	<i>This study</i>
ATEG_08450	GedB	β -lactamase type thioesterase	Heterologous expression, in vitro assays	[20]
ATEG_08451	GedC	Polyketide synthase	Deletion mutants, heterologous expression, in vitro assays	[19–21]
ATEG_08452	GedD	Putative O-methyl transferase*	BLAST, Hhpred	<i>This study</i>
ATEG_08453	GedR	Putative transcription factor	Deletion mutant, quantitative RT-PCR	<i>This study</i>
ATEG_08454	GedE	Putative glutathione-S-transferase	Annotation from BROAD, BLAST, Hhpred	<i>This study</i>
ATEG_08455	GedF	Putative oxidoreductase	BLAST, Hhpred	<i>This study</i>
ATEG_08456	GedG	Putative SAM-dependent-methyltransferase*	BLAST, Hhpred	<i>This study</i>
ATEG_08457-2	GedH	Putative emodin anthrone oxidase, similar to HypC	BLAST, 34% amino acid identity	<i>This study</i>
ATEG_08457-1	GedI	Putative mdpH homolog	BLAST, 46% amino acid identity	<i>This study</i>
ATEG_08458	GedJ	Dihydrogeodin oxidase	Enzymatic assays, protein sequencing	[18]
ATEG_08459	GedK	Putative Bayer Villiger-type oxidase	Enzymatic assays, Identity inferred in this study	[17]
ATEG_08460	GedL	Sulochrin halogenase	Deletion mutant, functional complementation	<i>This study</i>

All similarity percentages indicate identities at the amino acid level.

*One of these three putative ORFs is likely to encode the emodin O-methyltransferase described by Chen et al [16].

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annotated ATEG_08457.1) and ATEG_08457-2 (the new putative *HypC* homolog). Specifically, we suggest that ATEG_08457-2 and ATEG_08457-1 are positioned on *A. terreus* supercontig 12, base pairs 1307175–1307627 and 1308053–1308540, respectively.

Finally, we inspected the remaining ORFs in the region for activities relevant for production of geodin. Among these, one (ATEG_08454) was functionally annotated as a glutathione-S-transferase and two ORFs (ATEG_08455 and ATEG_08457-1) uncovered by the BLAST analysis displayed similarity to oxidoreductases and MdpH, respectively. The latter is a protein of unknown function required for emodin synthesis in *A. nidulans* [22]. To substantiate our predictions of the involvement of these putative genes, we conferred literature on similar biosynthetic pathways. The requirement for an oxidoreductase in geodin biosynthesis has previously been proposed by Henry and Townsend [26,27], while Simpson suggested the involvement of both a glutathione-S-transferase and an oxidoreductase in the biosynthesis of xanthenes in *A. nidulans* [28].

In addition to genes involved in the biosynthetic steps towards geodin, we noticed the presence of a gene, ATEG_08453, which encodes a putative transcription factor. The position of this gene within the putative geodin gene cluster suggests that it could regulate the activity of all genes in the cluster. In summary, our analysis suggests that the geodin gene cluster spans 25 kb and contains 13 putative ORFs (Figure 1A). Gene numbers, functional predictions and published data resulting from the entire analysis are presented in Table 1.

2.3 Strategy for transferring the geodin cluster from *A. terreus* into *A. nidulans*

Two vectors, containing 12 kb (Fragment 1) and 15 kb (Fragment 2) of the putative geodin gene cluster, respectively, were constructed by USER fusion by merging four individual PCR fragments in the first vector and seven PCR fragments in the second (Figure 1A). Assembling the geodin pathway from PCR fragments offers the possibility of introducing defined changes in the DNA sequence prior to integration via the many tools and methods for PCR based genetic engineering. In the present case,

we inserted the strong constitutive promoter, *P_{gpdA}*, of *A. nidulans* upstream of ATEG_08453, which encodes the putative transcription factor described above, with the intention that this modification would activate transcription of all genes in the geodin cluster after its integration into *IS1*. Importantly, a fragment of the geodin cluster (2 kb) is included in both constructs to serve as the upstream targeting sequence for the second gene-targeting event as illustrated in Figure 1B.

2.4 Production of geodin in *A. nidulans*

Using the two vectors constructed above, the putative geodin gene cluster (*ged*) was successfully transferred to an *A. nidulans* reference strain as well as to a strain where the entire monodictyphenone gene cluster (*mdpA-LΔ*) had been deleted. Geodin production in the *mdpA-LΔ* strains would indicate that all genes in the geodin cluster are functionally expressed, while geodin formation in the reference strain could be mediated via metabolites produced in the monodictyphenone pathway. The ability of the recombinant strains to produce geodin on minimal medium was analyzed by UHPLC-HRMS. The presence of geodin in fungal extracts was identified by comparison of retention time, accurate mass spectra, and isotope ratio to an authentic geodin standard. These analyses demonstrated that both *ged*⁺ and *ged*⁺ *mdpA-LΔ* produced geodin (Figure 3A). Consequently, we suggest that the putative transcription factor, ATEG_08453, is renamed *gedR* and that the enzymes in the cluster (ATEG_08449–08452 + ATEG_08454–08460) are renamed *gedA-L*. In different experiments, we observed that the amount of geodin is reproducibly higher in the *ged*⁺ strain (40–70 μg/plate) as compared to the amount in the *ged*⁺ *mdpA-LΔ* strain (2–4 μg/plate), which does not produce emodin via the *mdp* cluster. We therefore speculate that natively produced emodin, or other intermediates towards emodin production, could be converted into geodin in the *ged*⁺-strains.

2.5 Genetic characterization of the geodin cluster in *A. nidulans*

One reason for transferring a gene cluster into a host with a well-developed genetic toolbox is the possibility for further

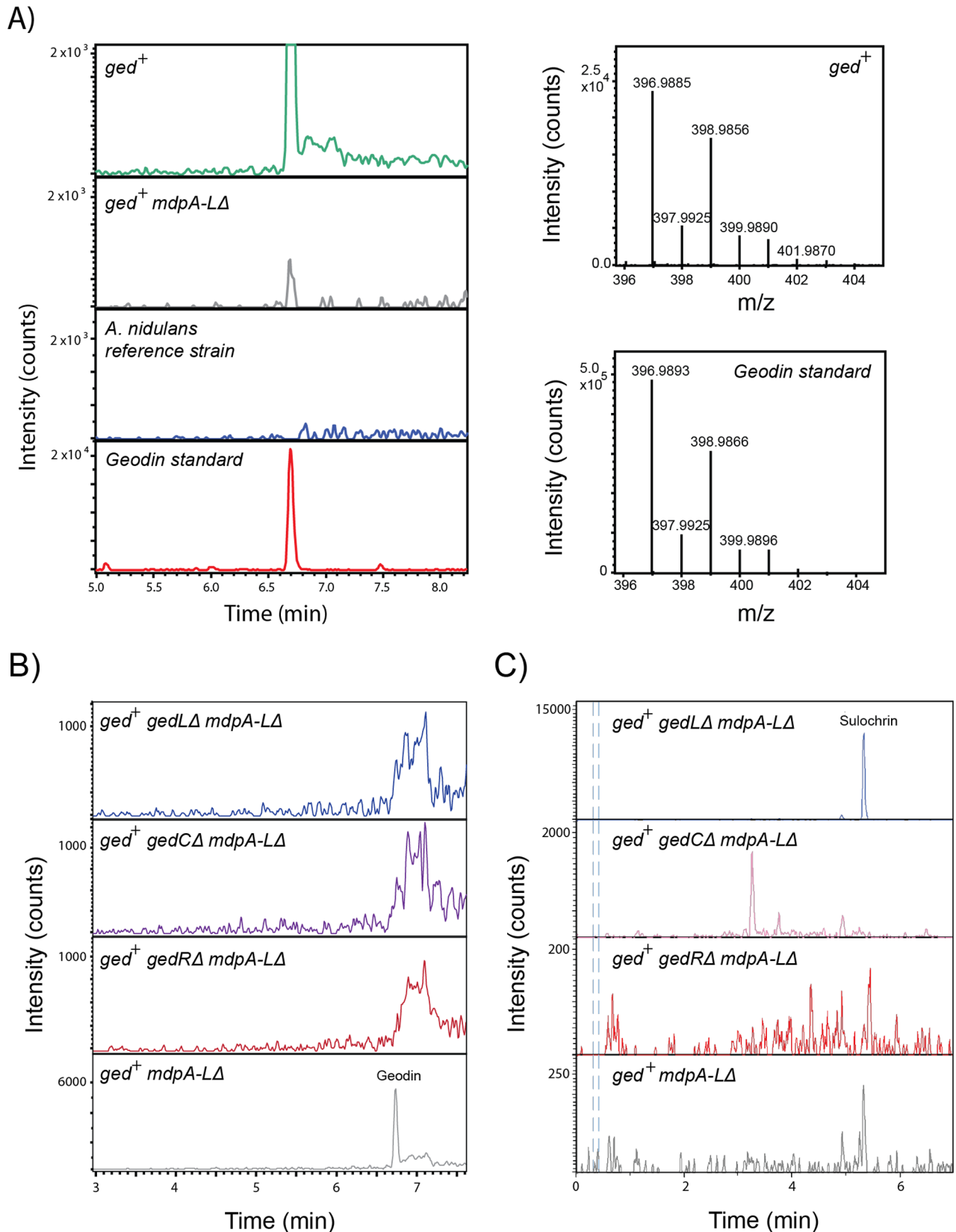


Figure 3. Production of geodin in *A. nidulans ged⁺* strains. **A)** Left panels depict extracted ion chromatograms (ESI-) of geodin m/z 396.9876 ± 0.005 amu from fungal extracts of *ged⁺*, *ged⁺ mdpA-LΔ* and reference strains (Bruker maXis system). An authentic geodin standard is included for comparison. The mass spectra of the putative geodin peak in *ged⁺* and the authentic geodin standard are depicted in panels to the right. **B)** and **C)** ESI- chromatograms of geodin m/z 396.9876 ± 0.005 amu (**B**) and sulochrin m/z 331.0812 ± 0.005 amu (**C**) extracted from *ged⁺ mdpA-LΔ* (grey), *ged⁺ mdpA-LΔ gedLΔ*, (blue), *ged⁺ mdpA-LΔ gedCΔ* (purple) and *ged⁺ mdpA-LΔ gedRΔ* (red).
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characterization of the cluster. To demonstrate this possibility, we decided to investigate the functionality of three key genes in the cluster, *gedC*, *gedR* and *gedL* encoding the PKS, the putative regulator, and the putative halogenase, respectively. We focused our efforts on the *ged⁺ mdpA-Δ* strains, as they provide a genetic background with no risk of complementation by *mdp* enzymes. UHPLC-HRMS analysis of strains grown on minimal medium revealed that all three deletion strains were unable to synthesize geodin (Figure 3B), thereby confirming that geodin is indeed produced from the reconstituted cluster and that the corresponding proteins of all three genes were functional in *A. nidulans* and play a role in geodin biosynthesis. We note the presence of a co-eluting isobaric compound, seen as the broad peak (6.7–7.3 min) in Figure 3B. However, this compound is not geodin as it does not contain a chlorine isotopic pattern. In agreement with previous analyses [19–21], no intermediates of the proposed geodin pathway (Figure 2) accumulated in the *ged⁺ gedCA mdpA-Δ* strain, which is expected, as the PKS responsible for geodin formation is absent.

According to the proposed biosynthetic route for geodin production, the halogenase accepts sulochrin as substrate and adds two chlorine atoms to form dihydrogeodin (Figure 2). Consistent with the hypothesis that *gedL* encodes the sulochrin halogenase, sulochrin accumulated significantly in the *ged⁺ gedLA mdpA-Δ* strain (1.2 – 1.8 μg/plate), but was undetectable in the *gedR* or *gedC* deletion strains (Figure 3C). To confirm that this lack of halogenase activity was due to the *gedL* deletion we reintroduced the *gedL* ORF at another ectopic site, *IS3*, which is a site located on a chromosome different from the one harboring *IS1*, see Figure S2. Surprisingly, no production of geodin was observed in this strain (Figure S3A). This prompted us to perform a BLAST search of the GenBank database [23] using the amino acid sequence of the current ATEG_08460.1 gene model as query. Strikingly, the majority of the best hits were enzymes that contain additional 49 amino acid residues in their N-terminus, including a conserved MSIP/MSVP motif at the very N-terminal end, see Figure S4A. Interestingly, intron prediction based on Augustus [29] predicts an intron just upstream of the AUG proposed by the current gene model (ATEG_08460.1). Taking this into account and by using an ATG further upstream in the *gedL* gene, a very similar extension can be generated for GedL, see Figure S4B and C. We therefore inserted a larger fragment of the *gedL* locus that includes this new ATG as well as its native UTR sequence into *IS2* [4] in the *ged⁺ gedLA mdpA-Δ* strain. In this strain, geodin was produced in ample amounts (4.0 – 6.8 μg/plate) strongly suggesting that *gedL* indeed encodes the sulochrin halogenase. Interestingly, in this strain, targeted analysis of the UHPLC-HRMS data and comparison to an in-house metabolite database [30], revealed 0.04 – 0.06 μg/plate of sulochrin and trace amounts of monochlor-sulochrin indicating that chlorine is added in two discrete catalytic steps, see Figure S3B.

To investigate whether GedR regulates the genes of the geodin cluster in *A. nidulans*, we performed a gene specific mRNA transcript analysis by quantitative RT-PCR in the *ged⁺ mdpA-Δ* and *ged⁺ gedRA mdpA-Δ* strains for all genes in the geodin gene cluster where a putative homolog is present in the monodictyphenone cluster (Table 1). This analysis demonstrated that transcription of all seven selected genes was down regulated in the absence of GedR. Most prominently transcription from four of the genes (*gedF*, *G*, *H*, and *K*) was reduced to less than 10% of the level obtained in the *ged⁺ mdpA-Δ* strain (Figure 4). We note that the de novo annotated candidate gene for the emodin anthrone oxidase, *gedH* (ATEG_08457-2), is transcribed in both the *ged⁺ mdpA-Δ* and *ged⁺ mdp⁺* strains. In addition, its expression levels in the two

strains were different from those obtained for *gedI* (ATEG_08457-1). Together, these observations strongly indicate that *gedR* encodes a transcription factor, which activates the expression of the genes that are involved in geodin synthesis and that *gedH* is a genuine ORF.

Inspired by these results, we next tested whether GedR would activate the *gedR* promoter. To this end we inserted a *lacZ* reporter gene under the control of the native *gedR* promoter into *IS3*, in *ged⁺ mdpA-Δ* and *ged⁺ gedRA mdpA-Δ* strains. On MM medium containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), colonies formed by the *P_{gedA}-lacZ* positive control strain were strongly blue, see Figure S5. The center of the colonies formed by *ged⁺ mdpA-Δ P_{gedR}-lacZ* strain exhibited slightly blue color. However, this level of blue represents background as it did not differ from the amount and location of blue color produced by the negative control strain *ged⁺ mdpA-Δ*, see Figure S5. In agreement with this, a quantitative RT-PCR analysis showed that the *lacZ* mRNA level was only modestly increased (1.5 fold) in the *ged⁺ mdpA-Δ P_{gedR}-lacZ* strain as compared to a *ged⁺ gedRA mdpA-Δ P_{gedR}-lacZ* strain, but this difference was not statistically significant ($p = 0.08$). Thus, GedR is not sufficient to induce expression from *gedR* in *A. nidulans*.

The fact that geodin production was significantly higher in the *ged⁺* than in the *ged⁺ mdpA-Δ* strains prompted us to investigate whether GedR could also activate transcription of the *mdp* cluster. Specifically, we compared transcription from *mdpG*, encoding the monodictyphenone PKS, in the *ged⁺* and the reference strains. In agreement with our hypothesis the *mdpG* transcript was easily detectable in the *ged⁺* strain, but undetectable in the reference, see Figure S6.

2.6 Conservation of gene clusters resembling the geodin cluster in other fungal species

Finally, we speculated whether gene clusters of a similar organization could be found in other sequenced fungal species as emodin is well-known to serve as precursor to a wide range of natural products [31–34]. Comparison of the geodin gene cluster to all *Aspergillus* genomes available at the *Aspergillus* Comparative Sequencing Project database (Broad Institute of Harvard and MIT, <http://www.broadinstitute.org/>) revealed the presence of putative gene clusters in *A. fumigatus* and *A. fischerianus* containing putative homologs of 12 of the 13 annotated ORFs in the geodin

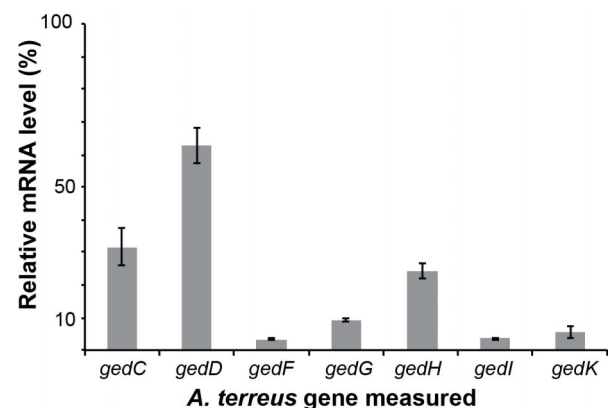


Figure 4. The *A. terreus* transcription factor GedR is important for gene expression in the geodin gene cluster in *A. nidulans*. Transcription levels of selected *ged*-genes in *ged⁺ mdpA-Δ*, *gedRA mdpA-Δ* strains relative to the corresponding levels in *ged⁺ mdpA-Δ* strains. doi:10.1371/journal.pone.0072871.g004

cluster (the halogenase, *gedL*, is absent). The internal organization of the putative clusters in *A. fumigatus* (Afu4g14450-14580) and *A. fischerianus* (101790-101920) were identical to the geodin cluster with the exception of an inversion affecting the five ORFs *gedG*-*gedK*. Moreover, the amino acid identities between biosynthetic enzymes in *A. terreus* and *A. fumigatus*/*A. fischerianus* were in average 58% and 60%, respectively. The conservancy across these three species further substantiates our delineation of the geodin cluster and hints that the putative clusters in *A. fumigatus* and *A. fischerianus* may encode the biosynthesis for a similar compound. Both species are known to produce trypacidin [35], which differs from geodin only by the absence of chlorines and the presence of an additional methyl group [36]. In agreement with the structural differences between geodin and trypacidin, the putative *A. fumigatus* and *A. fischerianus* gene clusters contain one additional putative methyltransferase, but lack the putative halogenase. Thus, the two putative clusters are candidates for trypacidin gene clusters.

Materials and Methods

Strains and media

Escherichia coli strain DH5 α was used to propagate all plasmids. Genomic DNA from the geodin producing *A. terreus* IBT15722 strain was used as template for PCR amplification of the geodin cluster. *A. nidulans* strains are shown in Table S1. *A. nidulans* strains were grown on solid glucose minimal medium (MM) prepared as described by Cove [37], but with 1 % glucose, 10 mM NaNO₃ and 2 % agar. MM was supplemented with 10 mM uridine (Uri), 10 mM uracil (Ura), and/or 4 mM L-arginine (Arg) when required. Solid plates containing 5-fluoroorotic acid (5-FOA) were made as MM+Uri+Ura medium supplemented with filter sterilized 5-FOA (Sigma-Aldrich) to a final concentration of 1.3 mg/ml.

Vector construction

All vectors were made by USER cloning and USER fusion [7,11]. All PCR products were amplified in 35 cycles using proof-reading PfuX7 polymerase [38]. Next USER fusions of vector and inserts were performed as previously described [12]. Reactions were incubated for 20 min at 37 °C, followed by 20 min at 25 °C before transformation into *E. coli*.

The pU2111-3 vector was constructed by USER fusion of 5 PCR amplified fragments: 1) vector backbone for propagation in *E. coli* (amplified with primers DH110/DH111), 2) US (upstream) targeting sequence for insertion in *IS3* (DH112/DH113), 3) PgpA-S::UEC::TrpC (DH114/DH115), 4) *A. fumigatus pyrG* (marker) (DH116/DH117) and 5) DS (downstream) targeting sequence for insertion in *IS3* (DH118-DH119). UEC: uracil excision cassette. Template for fragments 1 and 3: pU1111, for fragments 2 and 5: *A. nidulans* genomic DNA, and for fragment 4: pDEL2 [13]. p2110-3-*lacZ* was constructed by combining an AsiSI and Nb.BtsI pU2111-3 vector fragment with a PCR product containing the *E. coli lacZ* gene (amplified from pU2110-1-*lacZ* using motn136/motn137 [7] as primers) by USER cloning. The plasmid p2010-3-PgedR-*lacZ* was constructed by USER fusion of 5 PCR amplified fragments: 1) *gedR* promoter sequence (DH120/DH121, template: NID677 genomic DNA with), 2) *lacZ*::*TrpC*::*AFpyrG* (motn136/DH122, template: p2111-3), 3) vector backbone for propagation in *E. coli* (DH110/DH111 template: pU1111), 4) DS targeting sequence for insertion in *IS3* (DH123/DH119 template: pU2111-3) and 5) US targeting sequence for insertion in *IS3* (DH112/DH113). The vectors pU2110-3-ATEG_08460.1 and pU2110-2-*gedL* were constructed by combining the PCR

fragments generated with the primers JBN K35/K36 and JBN W77/W78 with pU2111-3 and pU2111-2 [4] vector fragments, respectively, by USER fusion. Prior to USER fusion both plasmids were digested with AsiSI and Nb.BtsI to create the vector fragments.

The two integration vectors containing the geodin gene cluster, pU1110-1-*ged1* (containing Fragment 1) and pU2000-*ged2* (containing Fragment 2), were made as follows. Primers for generating all PCR fragments for Fragment 1 and 2 assemblies are shown in Table S2. pU1110-1-*ged1* was constructed by combining all relevant fragments for Fragment 1 assembly into an AsiSI and Nb.BtsI pU1111-1 vector fragment by USER fusion. The vector fragment of pU2000-*ged2* is based on two PCR fragments generated by using primers 77/422 and 421/70 as well as pJ204 [7] and pU2111-1, respectively, as templates. These two PCR products and all relevant fragments for Fragment 2 assembly were then combined by USER fusion. The inserts in the two integration vectors were fully sequenced (StarSEQ, Germany).

Construction of *A. nidulans* strains

Protoplastation and gene-targeting procedures were performed as described by Nielsen et al [14] using either *argB* or *AFpyrG* as marker. All strains were verified by PCR analysis using spores as the source of DNA. Prior to PCR, the samples were incubated for 25 min at 98°C to liberate genomic DNA. This treatment was followed by touch-down PCR programs with annealing temperatures ranging from 64–56°C. Reactions were carried out in 35 cycles using 40 μ L volume with less than 1000 spore (one light stab in the colony with a pipette tip).

The *ged*⁺ (NID677) and *ged*⁺ *mdpA-LA* (NID695) strains were obtained by transformation of the relevant gene targeting substrates into NID74 [39] or NID356, respectively. The gene targeting substrates containing Fragment 1 and Fragment 2 were liberated from pU1111 (NotI) and pU2052 (SwaI), respectively, and gel purified (GFX™, GE Healthcare) prior to transformation. The resulting strains, NID677 and NID695, were subjected to counter selection on 5-FOA, generating NID802 and NID823, in order to recycle the *AFpyrG* marker, hence, allowing the use of this marker for subsequent gene deletions of *gedC*, *gedL* and *gedR*. These deletions were made as described in [14] using the primers listed in Table S2.

The strains NID1291 and NID1297, expressing *lacZ* under the control of ATEG_08453 promoter was made by transforming a gene targeting substrate liberated from pU2010-3-PgedR-*lacZ* by digestion with SwaI into NID823 and NID925, respectively. A control strain expressing *lacZ* under the constitutive promoter *PgpA* NID1278, was constructed by integrating the gene-targeting substrate liberated from pU2110-3-*lacZ* by SwaI. Both gene-targeting substrates integrate into *IS3* located between genes AN4770 and AN4769 on chromosome III, at position 1047840-1051735, by homologous recombination (See Figure S2).

The halogenase complementation strains, ATEG_08460.1 and *gedL*, were made in the NID1279 background, a pop-out recombinant strain from NID843. Digestion of vector pU2110-3-ATEG_08460.1 and pU2110-2-*gedL* liberated gene-targeting constructs for transformation and integration in *IS3* and *IS2*, respectively.

Chemical characterization of *A. nidulans* strains

All strains were grown as three point stab inoculations for 7 days at 37°C in the dark on solid MM-media. Extraction and analysis of

metabolites were performed by 2 methods: i) The agar plug extraction method described by Smedsgaard [40], using a total of 1 cm² of a colony, followed by analysis using reversed phase separation UHPLC-UV/VIS-HRMS on a maXis G3 quadrupole time of flight (qTOF) mass spectrometer (Bruker Daltonics, Bremen, Germany) connected to an Ultimate 3000 UHPLC system (Dionex, Sunnyvale, CA), and equipped with a 10 cm Kinetex C₁₈ column (Phenomenex Torrance, CA, USA) running a 10–100% acetonitrile gradient system in 10 min at 40°C; ii) more concentrated samples were made by extracting metabolites from a total of 15 cm² of a colony using 12 ml solvent (ethyl acetate-dichloromethane-methanol-formic acid 60:30:15:1 v/v/v) in a 16-ml vial. The extract was evaporated to dryness with N₂ flow and redissolved in 0.5 ml methanol and analyzed by reversed phase separation on Agilent 1290 UHPLC coupled to an Agilent 6550 qTOF (Santa Clara, CA, USA) equipped with an electrospray source, and equipped with a 25 cm Agilent Poroshell phenyl hexyl running a 10–100% acetonitrile gradient system in 15 min 60°C. Both MS instruments were mainly operated in ESI⁺ as geodin and related compounds ionizes best here in this mode [30]. Identification and quantification of geodin and sulochrin (BioAustralis, Smithfield, NSW, Australia) were based on comparison of peak area, retention time, accurate mass (± 1.5 ppm), isotope pattern and adduct pattern to quantitative authentic standards. Non quantitative standards representing 5-O-methylsulochrin; sulochrin-2'-methylether; isosulochrin; 3-O-demethylsulochrin; tryptacin; and emodin were also included in the analyses. Other intermediates were identified by comparison to an internal reference standard database (~1500 compounds) [30]. For the identification of geodin in NID695, high resolution MS (50 000 FWHM) and mass accuracy (< 1.5 ppm) of the maXis G3 was needed to exclude a non chlorine containing co-eluting isobaric compound, seen as the broad peak in Figure 3 (6.7–7.3 min) that impaired the identification of geodin. In the following strains geodin was further verified by better chromatographic separation on a 25 cm phenyl-hexyl column on the Agilent UHPLC-qTOF.

RNA isolation and quantitative RT-PCR

RNA isolation from the *A. nidulans* strains and subsequent quantitative RT-PCR reactions were done as previously described in [7] except that biomass for RNA isolation was prepared with a Tissue-Lyser LT (Qiagen) by treating samples for 1 min at 45 MHz. The *A. nidulans* histone 3 encoding gene, *hhtA* (AN0733) was used as an internal standard for normalization of expression levels. All primers used for quantitative RT-PCR are shown in Table S2.

Bioinformatic analysis of *gedL* (ATEG_08460.1)

Alignment illustrations and sequence were made in CLC Main Workbench 6.8.4.; Alignment parameters Gap open costs = 10, Gap extension cost = 1. All sequences in the alignments can be retrieved from Genbank, by the following references for the putative halogenases: *Aspergillus oryzae* ref|XP_001818590.1|, *Aspergillus terreus* ref|XP_001217599.1| and the *A. terreus* genome sequence resource at the Broad Institute, *Bipolaris sorokiniana* gb|EMD66881.1|, *Chaetomium chiversii* (*RadH*) gb|ACM42402.1|, *Chaetomium globosum* ref|XP_001227515.1|, *Pochonia chlamydosporia* (Rdc2) gb|ADM86580.1| [39] and *Talaromyces stipitatus* ref|XP_002486044.1|. Intron prediction of the *A. terreus* ATEG_08460.1 locus was done based on the Augustus gene prediction resource [29].

Concluding Remarks

We have described the complete and targeted transfer of all 13 genes of the geodin gene cluster from *A. terreus* to *A. nidulans* through a sequential integration approach enabling *A. nidulans* to synthesize geodin. In principle, this strategy can be used to reconstitute gene clusters of any size as the sequential integrations are based on marker recycling. In addition, defined promoters can easily be introduced in front of relevant genes in the cluster of interest. Importantly, we demonstrate that the cluster can be genetically dissected for clarification of its biochemical potential. We therefore envision that our method will significantly speed up the uncovering of biochemical pathways in fungi where the genome has been sequenced.

Supporting Information

Figure S1 Identification of putative HypC homolog encoded by *gedH* (ATEG_08457-2) in the *A. terreus* geodin gene cluster. Pairwise alignment of putative emodin anthrone oxidase, GedH (ATEG_08457-2), from *A. terreus* and norsolinic anthrone oxidase, HypC, from *A. flavus*. The conserved DUF-1772 domain and putative catalytic regions proposed by Ehrlich et al [25] are highlighted in green and blue, respectively. (TIF)

Figure S2 Schematic overview of the integration of a gene-expression cassette into the integration site, *IS3*, by homologous recombination. *IS3* is located between genes AN4770 and AN4769 on chromosome III. The cassette consists of six parts: upstream targeting sequence (US), promoter (P, in this case 0.5 kb *PgpdA*), your favorite gene (YFG), terminator (T, *TtpC*), marker (in this case *AFpyrG* flanked by direct), and the downstream targeting sequence (DS). The orientations of the genes AN4770 and AN4769 are indicated by green arrows. The sizes of US, DS and the intergenic region are 1984 bp, 1911 bp, and 3007 bp, respectively. (TIF)

Figure S3 Complementation of halogenase deficiency. **A)** Left panel: detection of sulochrin (-ESI, EIC(*m/z* 331.0812)); right panel: detection of geodin (-ESI, EIC(*m/z* 396.9876)). Strains for halogenase analysis, from top to bottom: NID843 (*gedLA*); NID1280 (*gedLA*, *IS3::PgpdA*-ATEG_08460.1); and NID1306 (*gedLA*, *IS2::gedL*). **B)** Ratio of geodin, monochlor-sulochrin, and sulochrin in the NID1306 strain, including ESI⁺-MS spectrum of monochlor-sulochrin showing the isotopic pattern and the mass deviations relative to the theoretical masses. Reference standards of geodin and sulochrin were included in all runs (data not shown). (TIF)

Figure S4 Identification of the likely start codon of *gedL*. **A)** Alignment of the top hits in a BLAST search for ATEG_08460.1 homologs shows that they contain a very conserved 48 amino acid residue addition in the N-terminus. Amongst the homologs, Rdc2, has been characterized as a halogenase by [39] MLAS is the predicted N-terminus of ATEG_08460.1. Drawing is not to scale. **B)** The position of putative exons and intron in the 5'-end of *gedL* as predicted by the Augustus software [29]. The predicted protein sequence encoded by exon 1 and by the first section of exon 2 is indicated. **C)** Full alignment of the halogenase homologs and GedL based on the GedL sequence derived from the new start codon. (TIF)

Figure S5 Expression of *lacZ* under the control of the *gedR* promoter (*PgedR*). Left panel: the positions of the strains

on the plate are shown in the right panel. NID823 (*ged*⁺ *mdpA*-*Δ*) is the reference strain without the *lacZ* gene. NID1278 is a control strain containing the *P_{gpdA}-lacZ* construct in *IS3*. The NID1291 (*ged*⁺ *mdpA*-*Δ* *P_{gedR}-lacZ*) strain carries *P_{gedR}-lacZ* in *IS3*. The strains were stabbed on MM containing X-gal and incubated three days at 37 °C in the dark before photography. (TIF)

Figure S6 Constitutive expression of *gedR* induces transcription of the *A. nidulans* gene *mdpG*. *mdpG* mRNA levels in reference (NID1) and in the *ged*⁺ strain (NID677) were evaluated by quantitative RT-PCR. For each strain, RNA was extracted as described in Materials and Method and the RNA samples analyzed in triplicate by quantitative RT-PCR. The samples were loaded and analyzed by 1% agarose gel-electrophoresis as indicated in the figure. (TIF)

Table S1 Strain genotypes. * = For reference see Nielsen et al ([13]). (XLSX)

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Supplementary

HypC	MEDLRDLSPRDLHKARGAKEEFKNVFLADGELTVHASFSRIFTRRTNKMGT EYVAVLTG	60
ATEG_08457-2	-----MA	2
HypC	SFLTGAMMNLHLLTIPILIETTRQPAQLVHQWSRIFYSGHRKGP GIALVTGALYGYA AWA	120
ATEG_08457-2	NPAVGAMMGLSLVAVPVFLDTNTQSEQLLAQFASLYDYG HKLMPTI AVATCALHGWVAAR	62
	. . .***. * *::: : : . * . ** : *:: : : *** : * ** : * *::: . *	
HypC	KYSVGE PWHHWMVAGVTTVSMVPYTWMFMNATNTALFHAEDQFEKGGVEISLQESVRLVG	180
ATEG_08457-2	KRAAHQ PWGRPVLA AVTTITMVPFTWCMVSTNNALFQLHK-----GADANMEMVRALIA	117
	* . : . : ** : : : * . *** : : ** : * : * : * : * : * : * : * : * : * : *	
HypC	KWDWLNTVRALFPLAGSVMGMLGVCGVVRY---	210
ATEG_08457-2	RWQWLHVARSLEPLAGAI VACQTLKELVGGSR	150
	: * : * : . . * : * * * * : : : . : : :	

Figure S1

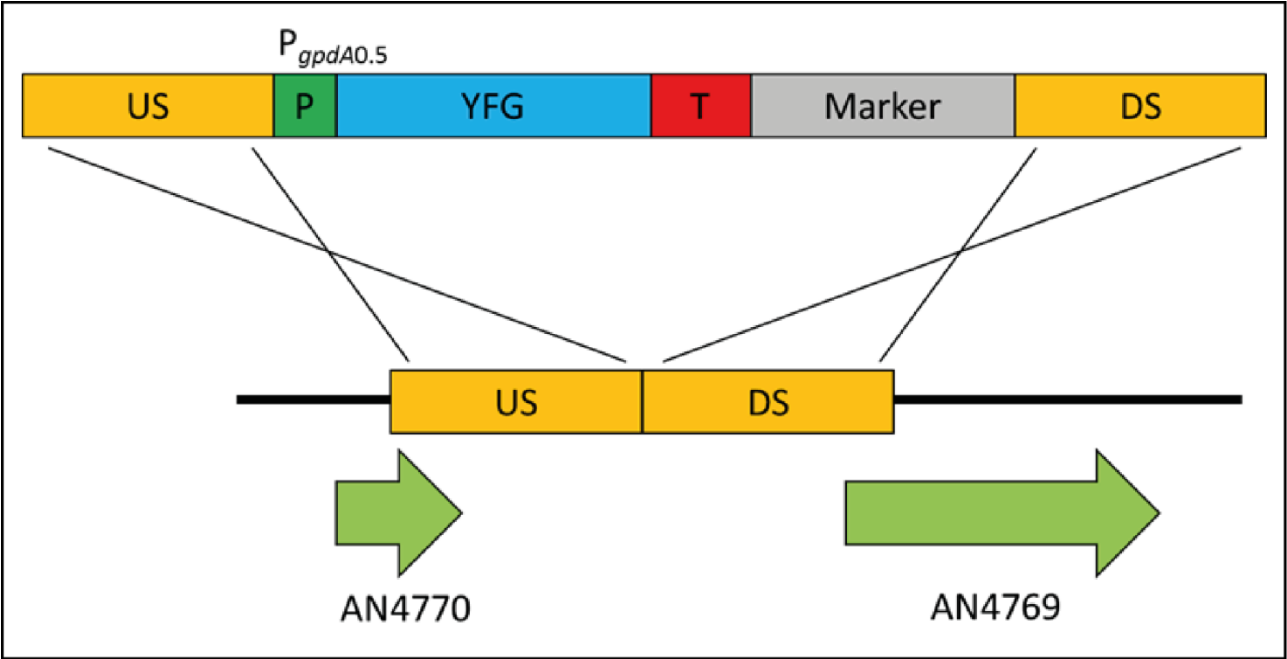


Figure S2

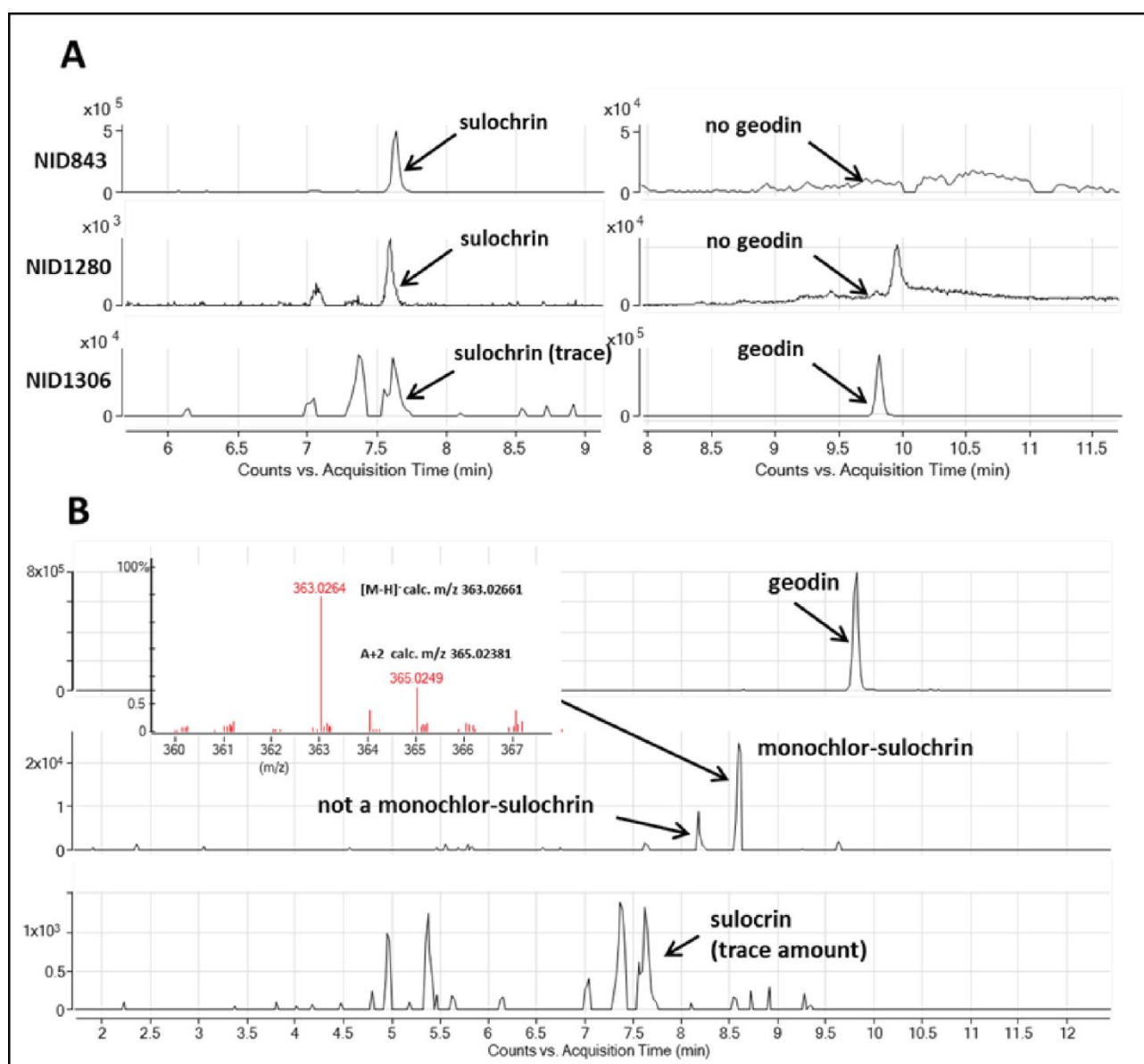


Figure S3

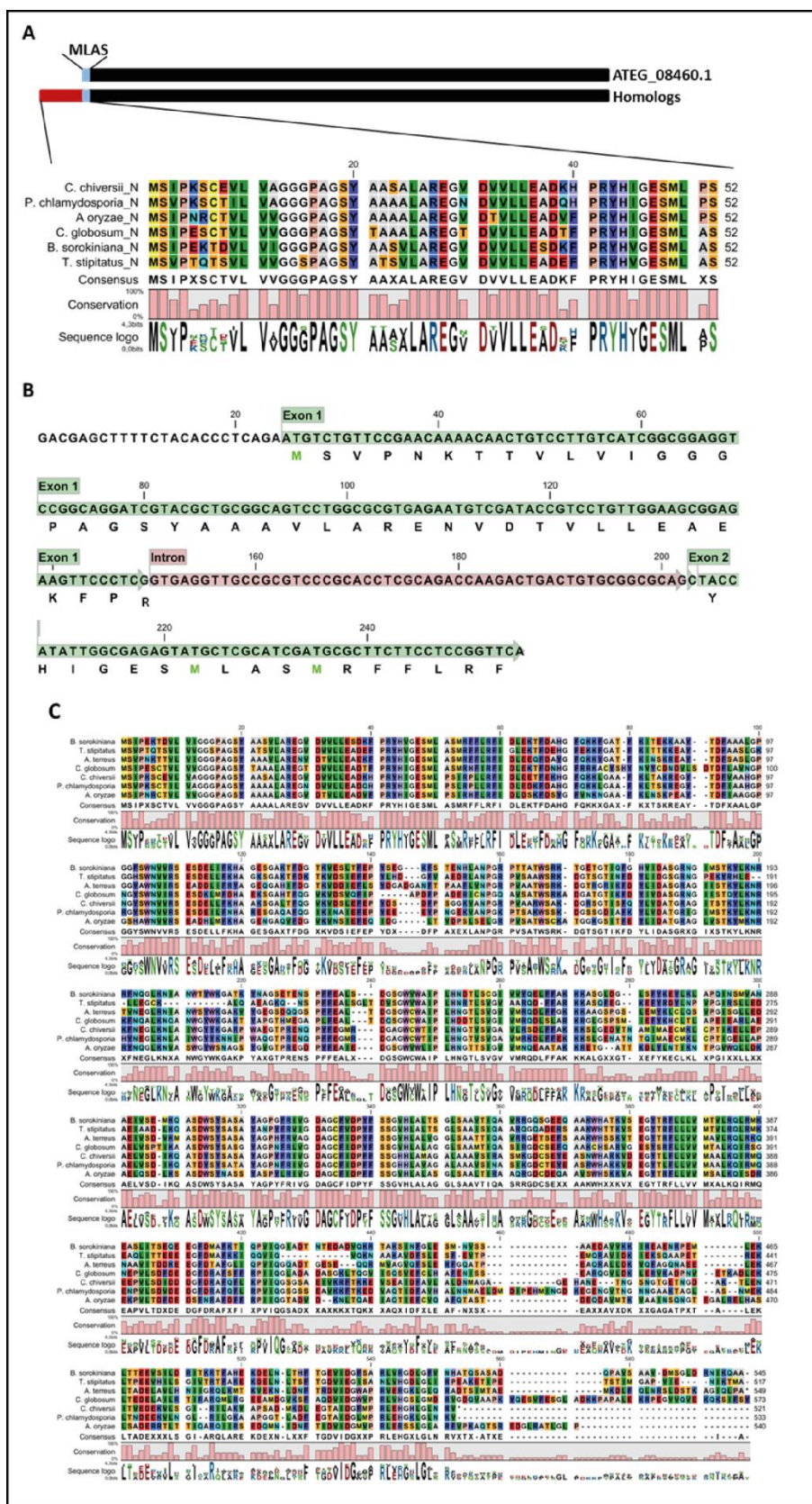


Figure S4

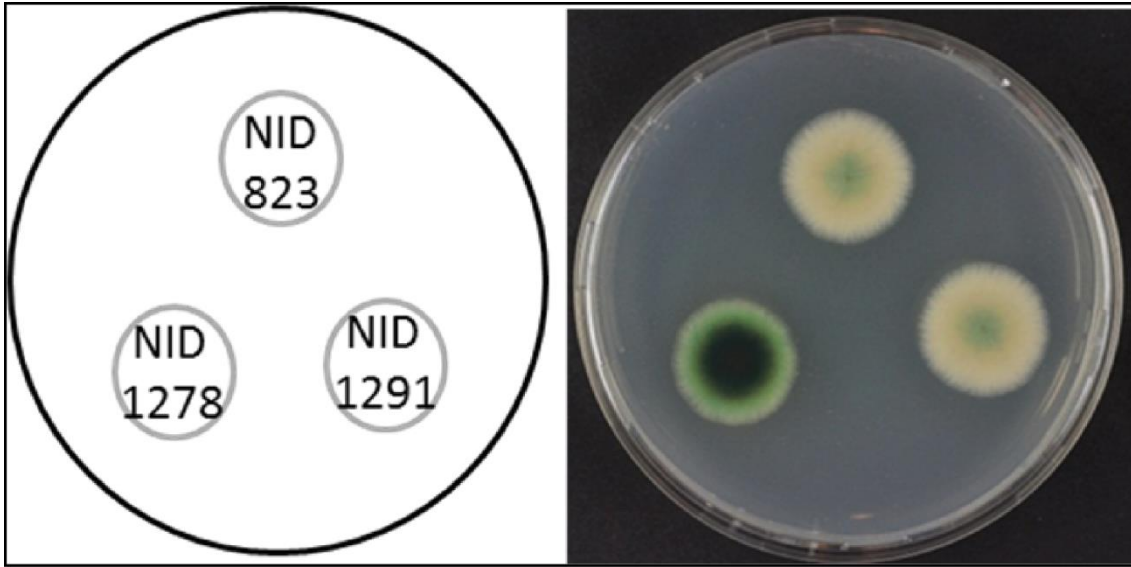


Figure S5

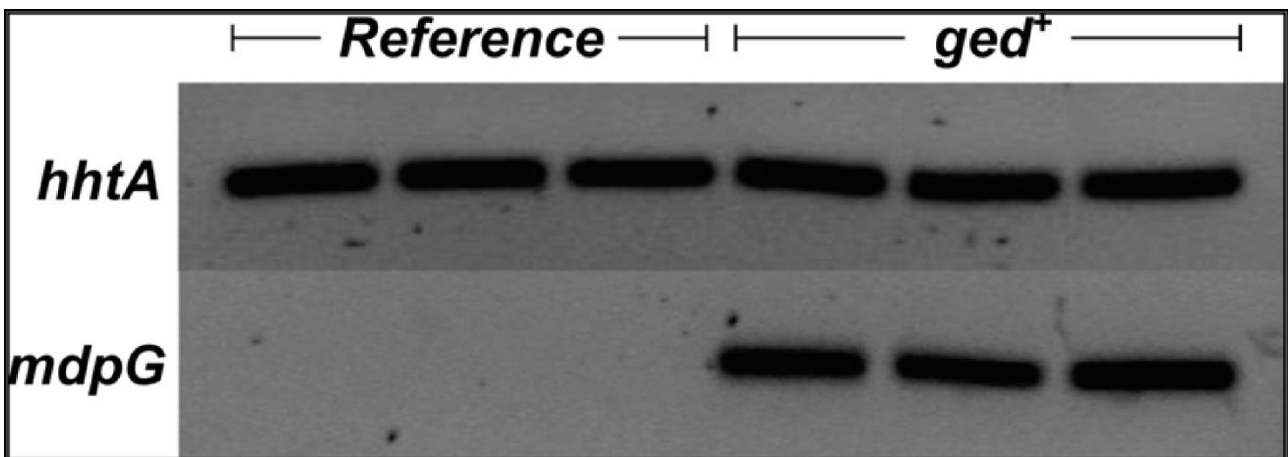


Figure S6

Table S1

Strain	Genotype
NID1	<i>argB2, pyrG89, veA1, nkuAΔ</i>
NID3	<i>argB2, pyrG89, veA1, nkuA-trS::AFpyrG</i>
NID74	<i>argBΔ, pyrG89, veA1, nkuAΔ</i>
NID356	<i>argBΔ, pyrG89, veA1, nkuAΔ, mdpA-LΔ</i>
NID675	<i>argBΔ, pyrG89, veA1, nkuAΔ, IS1::ATgedE-L::argB</i>
NID676	<i>argBΔ, pyrG89, veA1, nkuAΔ, mdpA-LΔ, IS1::ATgedE-L::argB</i>
NID677	<i>argBΔ, pyrG89, veA1, nkuAΔ, IS1::ATgedA-L::PgpdA-gedR::AFpyrG</i>
NID695	<i>argBΔ, pyrG89, veA1, nkuAΔ, mdpA-LΔ, IS1::ATgedA-L::PgpdA-gedR::AFpyrG</i>
NID761	<i>argBΔ, pyrG89, veA1, nkuAΔ, mdpA-LΔ IS1::ATgedA-L, gedRΔ:AFpyrG</i>
NID802	<i>argBΔ, pyrG89, veA1, nkuAΔ, IS1::ATgedA-L::PgpdA-gedR</i>
NID823	<i>argBΔ, pyrG89, veA1, nkuAΔ, mdpA-LΔ, IS1::ATgedA-L::PgpdA-gedR</i>
NID842	<i>argBΔ, pyrG89, veA1, nkuAΔ, mdpA-LΔ, IS1::ATgedA-B::gedD-gedL::PgpdA-gedR, gedCΔ:AFpyrG</i>
NID843	<i>argBΔ, pyrG89, veA1, nkuAΔ, mdpA-LΔ, IS1::ATgedA-K::PgpdA-gedR, gedLΔ:AFpyrG</i>
NID925	<i>argBΔ, pyrG89, veA1, nkuAΔ, mdpA-LΔ, IS1::ATgedA-L, gedRΔ</i>
NID1278	<i>argB2, pyrG89, veA1, nkuAΔ, IS3::PgpdA-lacZ</i>
NID1279	<i>argBΔ, pyrG89, veA1, nkuAΔ, mdpA-LΔ, IS1::ATgedA-K::PgpdA-gedR, gedLΔ</i>
NID1280	<i>argB2, pyrG89, veA1, nkuAΔ, IS1::ATgedA-K::PgpdA-gedR, gedLΔ, IS3::PgpdA-ATEG_08460.1::AFpyrG</i>
NID1291	<i>argBΔ, pyrG89, veA1, nkuAΔ, mdpA-LΔ IS1::ATgedA-L, IS3::0.9 kb PgedR-lacZ</i>
NID1297	<i>argBΔ, pyrG89, veA1, nkuAΔ, mdpA-LΔ IS1::ATgedA-L, gedRΔ, IS3::0.9 kb PgedR-lacZ</i>
NID1306	<i>argBΔ, pyrG89, veA1, nkuAΔ, mdpA-LΔ, IS1::ATgedA-K::PgpdA-gedR, gedLΔ:AFpyrG, IS2::gedL</i>

Table S2

PCR product #	Primer pair (fw/rv)	Forward primer	Reverse Primer	Description
1	455/456	agctcacuCCAGATCAACCCAGCAGACGA	aggccaggguGACGTTTTCGATTCCGCCCA	4.072 bp of <i>A. terreus</i> IBT15722 gDNA spanning putative ORFs ATEG_8454-8456 including 1788 bp overlap with 521/464 PCR product
2	457/177	acctgggcuCGTGA GTTGGGATGGAATGA	aacacaauGAAGGCTGTGTGATGCCCGG	4.382 bp of <i>A. terreus</i> IBT15722 gDNA spanning putative ORFs ATEG_8457-8458
3	178/508	attgtguAGATACCGCCAGAACTGCCTA	actggagauCGAGGATATGGAAGGACACTA	3.993 bp of <i>A. terreus</i> IBT15722 gDNA spanning putative ORFs ATEG_8459-8460
4	459/460	agctcacuTTAGAACCCAGCAGCAAGGGG	agcggaacuCCCTGTCGCTGCAGTTCAGTT	4.430 bp of <i>A. terreus</i> IBT15722 gDNA spanning putative ORFs ATEG_8449-8451 (5')
5	461/462	agtgctcgcuAACGCTTAGAGGCAAGCTACT	atctgcagacuGGATGACTAGATGGCACTGG	5.382 bp of <i>A. terreus</i> IBT15722 gDNA spanning the 3' end of the Geodin PKS (ATEG_8451)
6	463/522	agctctcagauAATGAGGTTACAAATGCATCC	agcttacuTGTGATGCTCCCTGGTGACT	2.397 bp of <i>A. terreus</i> IBT15722 gDNA spanning putative ORF ATEG_8452
7	405/406	agtaagcuCCCTAAATTGGCCCATCCG	atctgaaaauGCGGTAGTGATGTCTGCTCA	500 bp of <i>A. nidulans</i> <i>PgpdA</i> promoter amplified from pU1111 (20)
8	521/464	attttcagauGTCTGCAGAAAGGACACAAAGC	actggagauCATGGA TTTGCGGCAGTACT	2.533 bp of <i>A. terreus</i> IBT15722 gDNA spanning putative ORFs ATEG_8453-8454 including 1788 bp overlap with 455/456 PCR product
9	84/71	atctccaguTTACACAGACACGCAATCAT	agttagcuGCGGTTTTTTGGGGTAGTCA TC	<i>A. nidulans</i> cloning vector pU1111 (Hansen et al, 2011)
10	77/422	atctccaguuAGCGCGCGCCGCAATTTAAATC	acagagttcuTGAAAGTGGTGGGCTAACTAC	1.6 kb of basic cloning vector PJ204 (DNA 2.0) including the <i>amp^r</i> marker gene
11	421/70	agaactcguAGCACCGCCTACATACCTCG	agttagcuTGGATAACCGTATTACCGCCTTGG	5.3 kb of <i>A. nidulans</i> cloning vector pU2000 (nomenclature from (20)) including <i>A. fumigatus pyrG</i> marker gene flanked by direct repeats and <i>IS1</i> downstream targeting region
12	BGHA163/568	GGTCTACTCCCTGCGTCTA	CTCATCCAGCCTGTTATCCA	Analytical PCR for verification of insertion of fragment1 in <i>IS1</i> upstream targeting site
13	BGHA98/162	GGTTTCGTTGTCAATAAGGGAA	GTTCAGGGTGACGGTGAGAG	Analytical PCR for verification of insertion of fragment1 in <i>IS1</i> downstream targeting site
14	112/108	gggtttauuATGCTGCAGAAAGGACACAAAGC	gggtttauuATGACTACGACCTATGCAGT	Analytical PCR for verification of insertion of fragment2 in the overlap with the fragment1
15	BGHA 182/162	CACATCGACATCCTCACC	GTTCAGGGTGACGGTGAGAG	Analytical PCR for verification of insertion of fragment2 in <i>IS1</i> downstream targeting site
16	569/570	TCTGATCTGCTGGAGCCT	gatccccgggaattgcacatgCCGACTAGACGGTTGGATT	Deletion of the geodin polyketide synthase, ATEG_8451. Amplifies 2.0 kb of <i>A. terreus</i> IBT15722 gDNA upstream of the putative ORF. Specific tail for use of <i>A. fumigatus pyrG</i> as selective marker
17	571/572	aattccagctgaccaccatgACTGGTATCAGATTGGTCCG	CTTCAGGTGCGAGTAATCTT	As above, but 2.0 kb downstream of the putative ORF
18	573/574	CACGGA CGAGCATAGAAATAG	gatccccgggaattgcacatgACAGTCAGTCTTGTGCTGCG	Deletion of the putative sulochrin halogenase, ATEG_8460. Amplifies 2.0 kb of <i>A. terreus</i> IBT15722 gDNA upstream of the putative ORF. Specific tail for use of <i>A. fumigatus pyrG</i> as selective marker
19	J58/576	aattccagctgacacacatgTTACACAGACCAATCATC	AGAAAGAGGTCTGTGATGCTGA	As above, but 2.0 kb downstream of the putative ORF
20	JBN 5A/2K	catggcaattccgggagctCGATACCGCTATTACCGCC	GGAAAGAGAGTTCCACACC	Amplification of 1455 bp <i>A. fumigatus PyrG</i> 5' region for fusion PCR deletion substrates.

21	JBN 4Q/5B	TGATA CAGGTCTCGGTCCC	catgtgtgctcagtggaatttGCCAAGCTTAA CGCGTACC	Amplification of 1217 bp <i>A. fumigatus</i> <i>pyrG</i> 3' region for fusion PCR deletion substrates.
22	327/328	GAATCACTTGCCATCTTGCT	gatccccgggaattgcctgATTTCGACATAGCTCGTTG	Deletion of <i>A. nidulans</i> <i>mdp</i> gene cluster (<i>mdpA-L</i>) - upstream fragment
23	329/330	aattccagctgaccaccatgTGCTGTATATGGGTCTTGG	CTGTTTTGAGGCGGTCC	Deletion of <i>A. nidulans</i> <i>mdp</i> gene cluster (<i>mdpA-L</i>) - downstream fragment
24	BGHA 192/238	TCGTCAAGGTTTTCCCTTTGT	ACGCTATGTAGTGGTGAAT	Analytical PCR for verification of <i>mdpA-L</i> deletion
25	JBN 192/193	CCAGAGGCCAACCACTAC	CACCACTCGATTAGCTTGCC	Analytical PCR for verification of ATEG_8451 deletion
26	JBN 194/195	GGGAGCAGAAAGCAAGAG	CTCACTCGCTCTCGTTGC	Analytical PCR for verification of ATEG_8460 deletion
27	JBN 152/139	GTGCTCTCAGGAGTCCG	GAGGCGACGAGCAAGCTG	Expression analysis of <i>hhtA</i> by qRT-PCR
28	JBN K23/K22	TCCTCAAAACCCCTCCGCC	CTCCACCAGCTCCACAG	Expression analysis of ATEG_8452 by qRT-PCR
29	JBN 134/135	GGGACAAAGAGGTGGATGAG	GGTACAGGACGAGGTGC	Expression analysis of ATEG_8453 by qRT-PCR
30	JBN K20/K21	GGCGGTTATCGCTCGATG	GGTATGAGTGAAGAACGGG	Expression analysis of ATEG_8455 by qRT-PCR
31	JBN K18/K19	GCCCCATGACCAATCACC	CCAGTTCTCGATCTCTGCC	Expression analysis of ATEG_8456 by qRT-PCR
32	JBN 132/133	GGGCATGAGCGAGGAGGA	GAGTGA CGCGGGACTATG	Expression analysis of ATEG_8457-1 by qRT-PCR
33	JBN K16/K17	CCCTACCAACAATGCACGTGTTCC	CCCACCACAAAGCTCTTTC	Expression analysis of ATEG_8457-2 by qRT-PCR
34	JBN K13/K12	CTACATCTCCAAAGCTGCTC	CTCGGCTCTTCAGAGCCC	Expression analysis of ATEG_8459 by qRT-PCR
35	JBN R39/R40	GTTACTCAGGCCCTGTCTC	CCCGAAACTTCTCTGCTATCACCC	Expression analysis of <i>mdpG</i> by qRT-PCR
36	595/596	aattccagctgaccaccatgCTCCCTGCTGACTTGCCTAA	AACCTGTGTAAGCTTCTGCC	Deletion of the putative transcription factor ATEG_8453 - upstream fragment
37	593/594	TACGA GCCAAGGAGAACGAC	gatccccgggaattgcctgTGCTCTA TTGTGGTGTTCG	Deletion of the putative transcription factor ATEG_8453- downstream fragment
38	JBN K20/Q43	GGCGGTTATCGCTCGATG	CACCGATCTTCAGGATGACGC	Analytical PCR for verification of ATEG_8453 deletion
39	DH110/DH111	atccccacuACCGCA TTAAGACCTCAGCG	agctgctcuCGTCGA TTAACCCCTCAGCG	Amplification of vector backbone for propagation in <i>E. coli</i> including the ampR marker gene
40	DH112/DH113	aagcagcuCAAAGGTACCCCGCTACAGCA	atattgggcuAGCCTTCA TTGACCAGCTTCG	Amplification of IS3 upstream targeting sequence
41	DH114/DH115	agcccaatauTAAGCTCCCTAA TTGGCCC	agctcacuGGCGCTTACACAGTACA	Amplification of <i>A. nidulans</i> promoter <i>Pgpda</i> (498 bp), uracil excision cassette and <i>TtrpC</i> sequence (<i>Pgpda-S::UEC::TtrpC</i>)
42	DH116/DH117	agtgagcuTGGATAACCGTATTACCGCC	attactaguTGCCAAAGCTTAACGCGTAC	Amplification of <i>A. fumigatus</i> <i>pyrG</i> from pDel2 (Nielsen et al 2008)
43	DH118/DH119	actaggtauTGAATGTTGGAATAGGCTGTGC	agtggggauCTGCGATACCCGTCGTAGTCCT	Amplification of IS3 downstream targeting sequence
44	DH120/DH121	agcccaatauGAAAGTGGCTGATTTTGACGACTTG	atcgctcuTGTGATGCTCCCTGGTGAC	Amplification of 900 bp ATEG_8453 promoter (<i>PgedR</i>)
45	136/137	agagcgauATGACCATGATTACGGATTTC	tctgcgau TTA TTTTGTGACACAGACCA	Amplification of <i>lacZ</i> gene from (Hansen et al. 2011)
46	motni136/DH122	agagcgauATGACCATGATTACGGATTTC	aatgtaUGACGCCTTCTCCACAACA	Amplification of <i>lacZ::TtrpC::pyrG</i> sequence from pUJ2113
47	DH123/DH119	atacatuGTGCCTGTGCTATTAAACGATGAG	agtggggauCTGCGATACCCGTCGTAGTCCT	Amplification of IS3 downstream targeting sequence from pUJ2113
48	JBN W77/W78	agagcgauCTACCTGCTGCTGCGTAGC	tctgcgauGAGGATA TGGAAAGGACACTATG	Amplification of the extended ATEG_8460 ORF + flanks
49	JBN K35/K36	agagcgauATGCTCGCATCGATGCC	tctgcgauGGCGAGTAGCATAGCGG	Amplification of the original prediction of the ATEG_8460 ORF

2.4 Compartmentalization of monodictyphenone PKS and geodin PKS

2.4.1 Introduction

The reconstitution of the geodin biosynthetic pathway in *A. nidulans*, as described in section 2.3, showed that there was some overlap between the monodictyphenone pathway and geodin pathway. The amount of geodin produced, when the monodictyphenone biosynthetic cluster was present in *A. nidulans*, was higher than when the cluster had been deleted. As the pathway share some intermediates, we therefore speculated that the natively produced intermediates could be converted by the heterologous genes into geodin.

A common characteristic of SM metabolism is the compartmentalization of enzymes, intermediates and end products in sub-cellular membranous organelles such as vacuoles, vesicles and peroxisomes (Roze et al., 2011). Compartmentalization is a way for the cell to minimize diffusion and increase local enzyme and intermediate concentration as well as provide a means for the cell to sequester potentially toxic metabolites, which can be exported out of the cell without harming itself. The key role of peroxisomes for the production of fungal SMs have also been studied and a number of SMs are synthesized or partially synthesized in peroxisomes (Bartoszewska et al., 2011; Stehlik et al., 2014). Some of these known SMs are paxilline (Saikia and Scott, 2009) penicillin (Meijer et al., 2010) and AK toxins (Imazaki et al., 2010). To date, one of the best characterized pathways, with regards to localization of enzymes and intermediates in the cell, is the biosynthetic pathway of aflatoxin. The model for aflatoxin biosynthesis suggests that the initial steps of aflatoxin takes place in the peroxisomes, thereafter, specialized multifunctional vesicles, called aflatoxisomes, carry out the rest of the biosynthesis and export of the product (Maggio-Hall et al., 2005; Chanda et al., 2009).

2.4.2 Results and discussion

In order to investigate if the monodictyphenone PKS (MdpG) and geodin PKS (GedC), are localized together in the same compartments, the PKSs were tagged with fluorescent proteins. MdpG was fused C-terminally with mRFP and GedC was similarly C-terminally fused with GFP and expressed under the constitutive *gpdA* promoter. These constructs were transformed into a strain, where the native mdpG gene was knocked out in order to verify that the MdpG-RFP fusion protein is functional. HPLC-MS analysis showed that the MdpG-RFP fusion protein was active as emodin, a product based on the PK backbone produced by MdpG, is observed in the sample (Kleijnstrup et al., 2012) (see Figure 2.1).

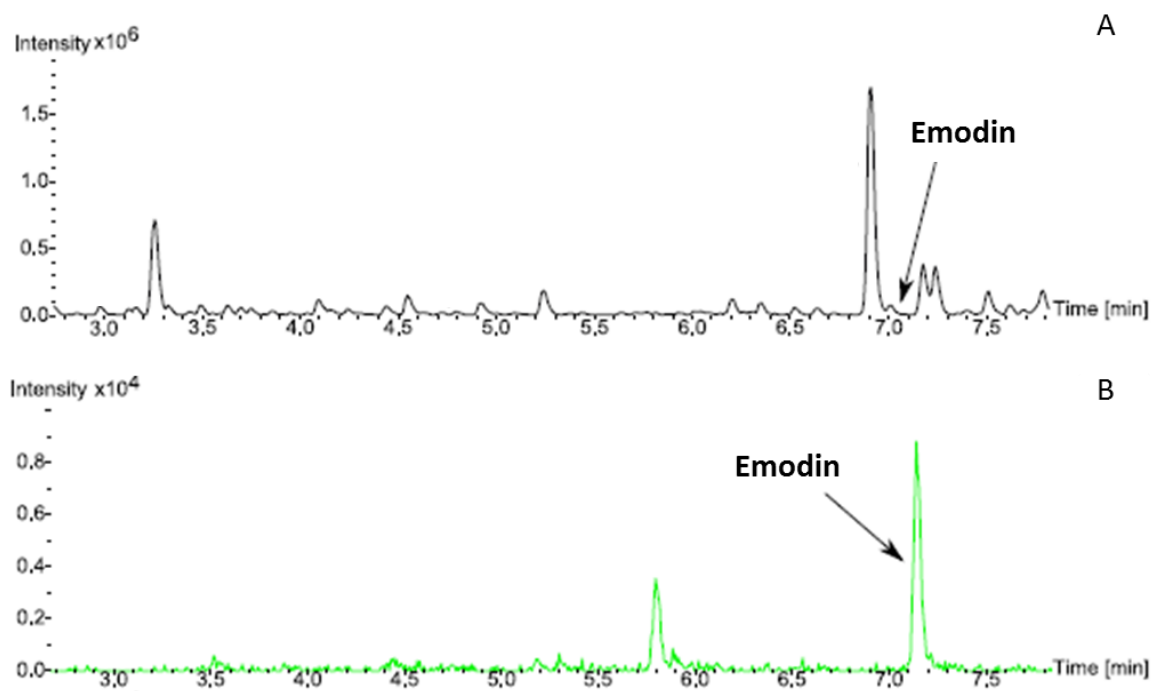


Figure 2.1: Chromatograms of the strain with overexpressed *mdpG-rfp* after growth on CYA with supplements for seven days. (A) Base peak chromatogram. Emodin is highlighted. (B) Extracted ion chromatogram (EIC) of the mass 271.0601 (± 0.001), corresponding to emodin

Fluorescence microscopy of the strain with the MdpG-RFP and GedC-GFP fusion proteins clearly indicate that they are co-localizing to the same compartment or that they might be aggregating together (see Figure 2.2). If they are co-localizing to a compartment, this could facilitate easy access to intermediates generated from the different pathways and be a part of the reason, why higher geodin production was achieved, when the monodictophenone (*mdp*) pathway was present.

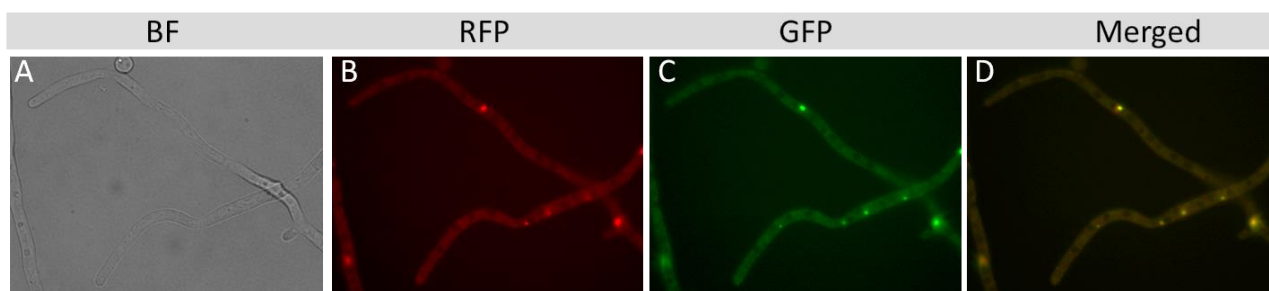


Figure 2.2: Bright field and fluorescence pictures of the strain with MdpG-RFP and GedC-GFP grown for 24 hours at 30°C. (A) Bright field, (B) RFP filter, (C) GFP filter and (D) B and C merged.

To assess whether the MdpG-RFP localizes to the peroxisomes, the *mdpG-rfp* was co-expressed with GFP containing a C-terminal extension with the peroxisomal targeting signal type 1 (PTS1). Fluorescence

microscopy indicated that some of the signal from the MdpG-RFP overlaps with the signal from the peroxisome localized GFP-PTS1. This could suggest that MdpG-RFP in part localizes in the peroxisomes or that MdpG-RFP localizes to specialized peroxisomes (see Figure 2.3). Even though the MdpG was predicted to contain neither PTS1 or PTS2 (<http://mendel.imp.ac.at/pts1/> and <http://psort.hgc.jp/form2.html>), it is possible the protein is transported to the peroxisomes, as proteins lacking both signals have successfully been transported to the peroxisomes (Klein et al., 2002).

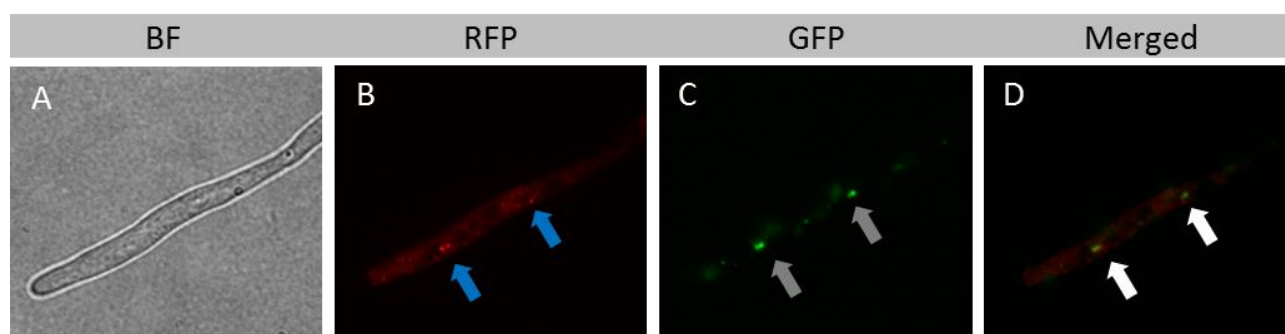


Figure 2.3: Bright field and fluorescence pictures of the strain with MdpG-RFP and GFP-PTS1 grown for 24 hours at 30 °C. (A) Bright field, (B) RFP filter, (C) GFP filter and (D) B and C merged. Blue arrows indicate localization of MdpG-RFP. Gray arrows indicate peroxisomes of interest. White arrows point out areas, where peroxisomes and MdpG co-localize.

2.4.3 Concluding remarks

These results suggest that the MdpG and GedC are compartmentalized in the same compartment. Further work is required to identify which specific compartment they both localize to, though there are some indications that the peroxisomes might be involved. Furthermore, in this study the expression of the genes were under the regulation of the strong constitutive promoter, which may not be the most optimal set-up for the study of compartmentalization, thus, further studies using the native promoter or a tunable inducible promoter (e.g. Tet-on system), will be used to better mimic the natural conditions.

Gaining more understanding of the compartmentalization of both the geodin pathway as well as the monodictophenone/emodin pathway may give insight to whether the whole pathway is compartmentalized in the same compartment or, as in the case of aflatoxin, involve multiple compartments and if the pathways differ. Knowledge of the regulation and compartmentalization of these pathways may facilitate compartment specific targeted expression of SMs genes and increase the production of compounds of interest.

2.4.4 Material and Methods

Strains and media

The *A. nidulans* strain strain IBT 30995 (*argB2*, *pyrG89*, *nkuAΔ*, *veA1*, *mpdGΔ*) (Nielsen et al., 2011) was used for strain construction. Plasmids were propagated in *E. coli* strain DH5α. *A. nidulans* strains were grown on minimal medium (MM) containing 1% glucose, 0.001 % thiamine, 0.1 % trace element solution and 5 % nitrate salts solution. For solid medium 2 % agar was added. Nitrate salts solution (per Liter): 120 g NaNO₃, 10.4 g KCl, 10.4 g MgSO₄·7H₂O, 30.4 g KH₂PO₄. Trace element solution (per Liter): 0.4 g CuSO₄·5H₂O, 0.04 g Na₂B₄O₇·10H₂O, 0.8 g FeSO₄·7H₂O, 0.8 g MnSO₄·2H₂O, 0.8 g Na₂MoO₄·2H₂O, 8 g ZnSO₄·7H₂O. MM was supplemented with 10 mM uridine (Uri), 10 mM uracil (Ura) and/or 4 mM L-arginine (Arg), when necessary. Solid medium containing 5-fluoroorotic acid (5-FOA) was made as MM+Ura+Uri+Arg supplemented with 1.3 mg/mL 5-FOA.

PCR and vector construction

All PCR products were amplified using proofreading PfuX7 polymerase (Nørholm, 2010) in 35 reaction cycles with 60 °C annealing temperature. *mdpG* was amplified from *A. nidulans* IBT 29539 gDNA (Nielsen et al., 2008) with primers N44 and N45 and *gedC* was amplified from *A. terreus* IBT 15722 gDNA with primers Q41 and DC261. *rff* was amplified from pU2110-1-*rff* using primers C83 and C54. *gfp* was amplified from pU2310-1-*gfp* with Z43 and M55 for tagging of *gedC* and T80 and DC231 for *gfp*-PTS1. See Table 2.1 for a list of all primers used. The PCR fragments were inserted into plasmid pU2111-2 for insertion in IS2 (Hansen et al., 2012). The plasmid was prepared for USER cloning by digesting with the respective restriction and nicking enzymes, and the cloning procedure was as described in (Nour-Eldin et al., 2006). Plasmids were verified by restriction analysis and linearized by digestion with *Swa*I prior to transformation into *A. nidulans* IBT 30995.

Strain construction

Protoplastation and transformation of *A. nidulans* were performed as described (Johnstone et al., 1985; Nielsen et al., 2006) using *AFpyrG* as a selection marker. All transformants were verified by diagnostic PCR. Strains transformed with *AFpyrG* were subjected to counter selection on MM+Ura+Uri+Arg +5-FOA in order to recycle the *AFpyrG* marker. All strains used in this study are listed in Table 2.2.

Fluorescence microscopy

MM agar slides were prepared by pipetting 1 ml MM with agar on a slide. MM agar slides were inoculated with spores and grown 24 hours at 30°C in petri dishes. Live cell images were captured with a cooled Evolution QEi monochrome digital camera (Media Cybernetics Inc.) mounted on a Nikon Eclipse E1000 microscope (Nikon).

Table 2.1. List of primers used in this study

Name	Sequence
N44	AGAGCGAUATGCCGTATATACTCCTC
N45	AGGAGGCCAUATTATAGTACTCTAATAGCCAGCT
Q41	AGAGCGAUATGGATTTCACCCCGTCG
DC261	ATACCGGAUCCGCTGTAATATTCTAGAAGCCAGC
C83	ATGGCCTCCUCCGAGGACG
C54	TCTGCGAUTTAGGCGCCGGTGGAGTG
Z43	ATCCGGTAUGGTGAGCAAGGGCGAG
M55	TCTGCGAUTTACTTGTACAGCTCGTCCATGC
T80	AGAGCGAUATGGTGAGCAAGGGCGAG
DC231	TCTGCGAUTCACAGCTTGGACTTGTACAGCTCGTCCATGC

Table 2.2. List of strains used in this study

Strain	Genotype	Source
IBT 30995	<i>argB2, pyrG89, nkuAΔ, veA1, mpdGΔ</i>	(Nielsen et al., 2011)
MpdG-RFP,GFP-PTS1	<i>argB2, pyrG89, nkuAΔ, veA1, mpdGΔ, IS1::PgpdA::mdpG-RFP::TtrpC, IS2::PgpdA::GFP-PTS1::TtrpC::pyrG</i>	This study
MpdG-RFP,GFP-PTS1	<i>argB2, pyrG89, nkuAΔ, veA1, mpdGΔ, IS1::PgpdA::mdpG-RFP::TtrpC, IS2::PgpdA::gedC-GFP::TtrpC::pyrG</i>	This study

3 Glycosylation

3.1 Elucidation of mannosyltransferase activity in the ER of *Aspergillus nidulans*

3.1.1 Introduction

Filamentous fungi from the genus *Aspergillus* are important industrially for the production of organic acids and enzymes (Bhat, 2000; Polizeli et al., 2005; Goldberg et al., 2006; Pel et al., 2007). The high secretion capacity of these fungi combined with the ability for posttranslational processing of proteins, such by glycosylation, have also made them attractive as hosts for production of heterologous proteins (Demain and Vaishnav, 2009; Sharma et al., 2009; Lubertozi and Keasling, 2009).

Glycosylation is one of the most common posttranslational modifications. It is estimated that approximately 50% of all eukaryotic proteins are glycosylated (Apweiler, 1999), though this ratio varies depending on the organism (Zielinska et al., 2012). Glycosylation plays an important role in protein folding, protein secretion and function, cell wall synthesis and cell signaling (Helenius, 2001; Helenius and Aebi, 2004; Mitra et al., 2006; Zhao et al., 2008; Jin, 2012; Beckham et al., 2012).

Glycosylation is also important in the production of therapeutic proteins as approximately 70% of the approved therapeutic proteins are glycosylated (Sethuraman and Stadheim, 2006). Glycosylation is involved in protein stability, ligand binding, immunogenicity and serum half-life (Li and d'Anjou, 2009). A major drawback in the production of therapeutic glycoproteins in *Aspergillus* is the difference in the glycosylation mechanism between mammalian cells and fungi. While the main form of asparagine (N) linked glycans attached to proteins in filamentous fungi are of the high-mannose type, the mammalian cells attach complex N-glycans with terminal sialic acid residues (Brooks, 2004; De Pourcq et al., 2010).

The biosynthesis pathway of N-glycans in the endoplasmic reticulum (ER) is conserved in all eukaryotes, but the pathway diverges when the glycoprotein is transferred to the Golgi and modified. The N-glycosylation pathway in filamentous fungi is not as well characterized as the mammalian or yeast pathway (Kornfeld and Kornfeld, 1985; Marth and Grewal, 2008; Hamilton and Gerngross, 2007), but the pathway has been predicted by comparative genomics and proteomics (Deshpande et al., 2008; Geysens et al., 2009). However, few genes of the pathway have been studied in filamentous fungi (Kainz et al., 2008; Kotz et al., 2010; Maddi and Free, 2010; Motteram et al., 2011; Dai et al., 2013; Chen et al., 2014b).

Glycoengineering in the yeast *Pichia pastoris*, has facilitated the production of complex bi-antennary human-like glycan structure, Sia₂Gal₂GlcNAc₂Man₃GlcNAc₂ in yeast (Choi et al., 2003; Hamilton and Gerngross, 2007; Jacobs et al., 2009). Thus, making it possible to produce erythropoietin and IgG with

human-like glycan structures (Hamilton et al., 2006; Ha et al., 2011). In contrast, far less progress has been made in humanizing the N-glycans from filamentous fungi (Kalsner et al., 1995; Maras et al., 1999; Kasajima et al., 2006; Kainz et al., 2008).

Kainz and co-workers deleted the dolichyl-P-Man:Man₅GlcNAc₂-PP-dolichyl α -1,3 mannosyl-transferase (*alg3*) gene in *A. nidulans* and *A. niger*. The enzyme encoded by this gene incorporates the first dolichyl-P-man-derived mannose in an α -1,3-linkage into the Man₅GlcNAc₂-PP-Dol inside the endoplasmic reticulum (ER). Thus, it is an enzyme involved in the early steps of N-linked glycan synthesis. Deletion of the gene generates an intermediate, which can subsequently be used as a precursor for generating complex glycans (Hamilton and Gerngross, 2007) (see Figure 3.1 A). Furthermore, in *Aspergilli* the Man₅GlcNAc₂ can be trimmed by α -1,2 mannosidases to Man₃GlcNAc₂, which also functions as a substrate for GNT I and GNT II (Bobrowicz et al., 2004; Parsaie Nasab et al., 2013). Analysis showed that the glycans produced from this mutant consisted mainly of the types with mass corresponding to Man₃₋₆GlcNAc₂ and a less abundant group with a mass corresponding to Man₇₋₈GlcNAc₂ or Glc₁₋₂Man₅GlcNAc₂. The latter glycan structure is due to incomplete processing of the glycan in the ER. Almost all of the structures could be reduced by α -1,2 mannosidases.

The objective of this study was to elucidate where the elongation of the Man₅GlcNAc₂ takes place. Identifying where the transferase activity takes place and the enzymes responsible for this activity will enable the generation of a more homogenous glycan pattern, which can function as precursors for further humanization of the pathway in *A. nidulans*. Thereby, allowing the exploitation of the high secretion capacity of these fungi for the production of therapeutic proteins.

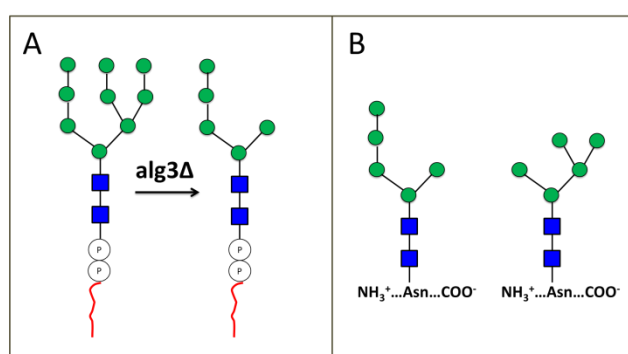


Figure 3.1: (A) Deletion of *alg3* truncates the dolichol linked glycan precursor, which is transferred to the protein. (B) Possible different glycan forms of Man₅GlcNAc₂ observed in the deletion mutants.

Results and discussion

3.1.2 *alg3* gene deletion and localization

As previously mentioned Alg3 is the first enzyme catalyzing the transfer of mannose to $\text{Man}_5\text{GlcNAc}_2\text{-PP-Dol}$ generating $\text{Man}_6\text{GlcNAc}_2\text{-PP-Dol}$ inside the lumen of the ER. The α -1,3-mannosyltransferase gene *AN0104* gene sequence was found at the *Aspergillus* Genome Database (AspGD). To verify that the enzyme is located in the ER the Alg3 was C-terminally fused with mRFP. Fluorescence microscopy indicates that the Alg3 localizes to the membrane of an organelle (see Figure 3.2). The observed pattern is similar to the ER pattern, which has been previously reported (Watanabe et al., 2007; Wang et al., 2010; Markina-Iñarrairaegui et al., 2013). Thus, it is very likely that Alg3 is localized to the ER membrane.

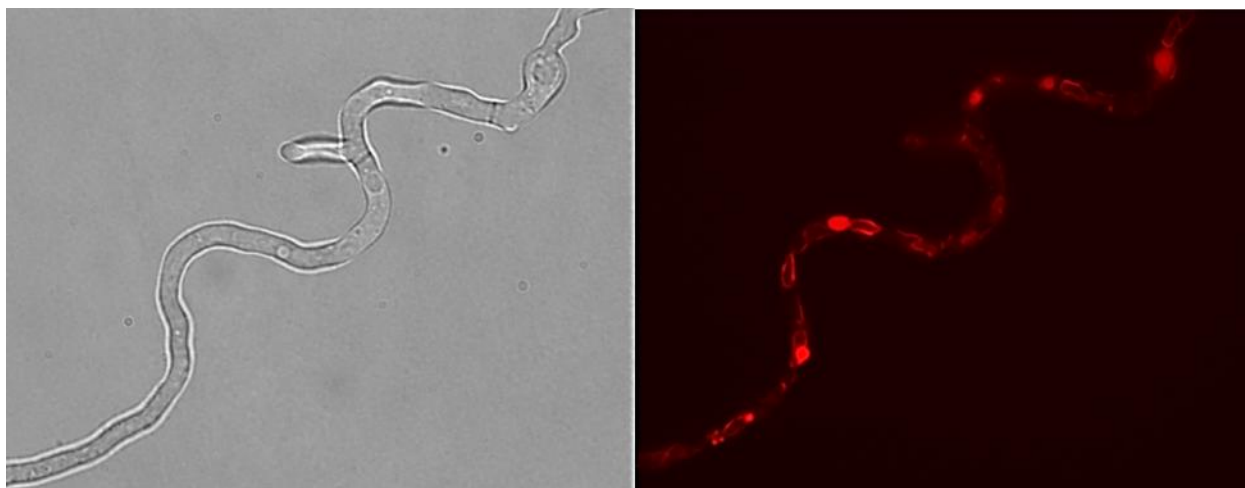


Figure 3.2: Localization of Alg3-mRFP fusion protein.

Subsequently the *alg3* gene was deleted and analysis of the glycans attached to a secreted β -glucosidase from the *alg3* Δ mutant shows, as previously observed (Kainz et al., 2008), that there is a shift towards a lower glycan profile from $\text{Man}_{5-10}\text{GlcNAc}_2$ to the range of masses corresponding to $\text{Man}_{3-7}\text{GlcNAc}_2$ (see Figure 3.3B). In addition, the elution time of peaks with the same mass is changed between the spectrum from the reference strain and the *alg3* Δ strain. It is also apparent that the peaks with masses corresponding to $\text{Man}_5\text{GlcNAc}_2$ and $\text{Man}_7\text{GlcNAc}_2$ are doublets, indicating that two isomers with the same mass are present. Hence, the glycan forms generated by this deletion are different from the reference strain, though they have the same mass. The presence of two isomers of $\text{Man}_5\text{GlcNAc}_2$ could suggest that the truncated $\text{Man}_5\text{GlcNAc}_2$, from the *alg3* Δ mutant, is first trimmed to $\text{Man}_{3-4}\text{GlcNAc}_2$ and thereafter elongated by the action of mannosyltransferases. This could generate a different isomer of $\text{Man}_5\text{GlcNAc}_2$ resulting in the observed doublet (see Figure 3.3B).

Deletion of all mannosyltransferases in the ER

The fact that the glycan profile of the *alg3Δ* mutant contains structures with a higher mass than $\text{Man}_5\text{GlcNAc}_2$ lead us to investigate if any or some of the mannosyltransferase activity observed in the *alg3Δ* mutant takes place in the ER. The genes of the two remaining known ER mannosyltransferases, α -1,2-mannosyltransferase (*alg9*) and α -1,6-mannosyltransferase (*alg12*) involved in the formation of the glycan precursor were deleted. The genes were identified by a BLAST search using the amino acid sequence of *S. cerevisiae* Alg9p (YNL219C) and Alg12p (YNR030W) as a query against all *A. nidulans* sequences in AspGD. Accordingly, the putative genes *AN10118* and *AN3588* coding for the enzymes with α -1,2-mannosyltransferase and α -1,6-mannosyltransferase activity, respectively, were identified. The glycan analysis showed that the glycan profile of the *alg3Δalg9Δalg12Δ* mutant did not change significantly when compared with the single *alg3Δ* deletion strain (see Figure 3.3C). It can therefore be inferred that the mannosyltransferases encoded by the genes in the ER, do not use the $\text{Man}_5\text{GlcNAc}_2\text{-PP-Dol}$ as a substrate and the mannosyltransferase activity was a product of one or more Golgi localized mannosyltransferases. This also corresponds to what has been observed in *S. cerevisiae*, where the function of one of the Golgi localized mannosyltransferases accounted for some of the mannosyltransferase activity on a truncated dolichol linked glycan (Parsaie Nasab et al., 2013).

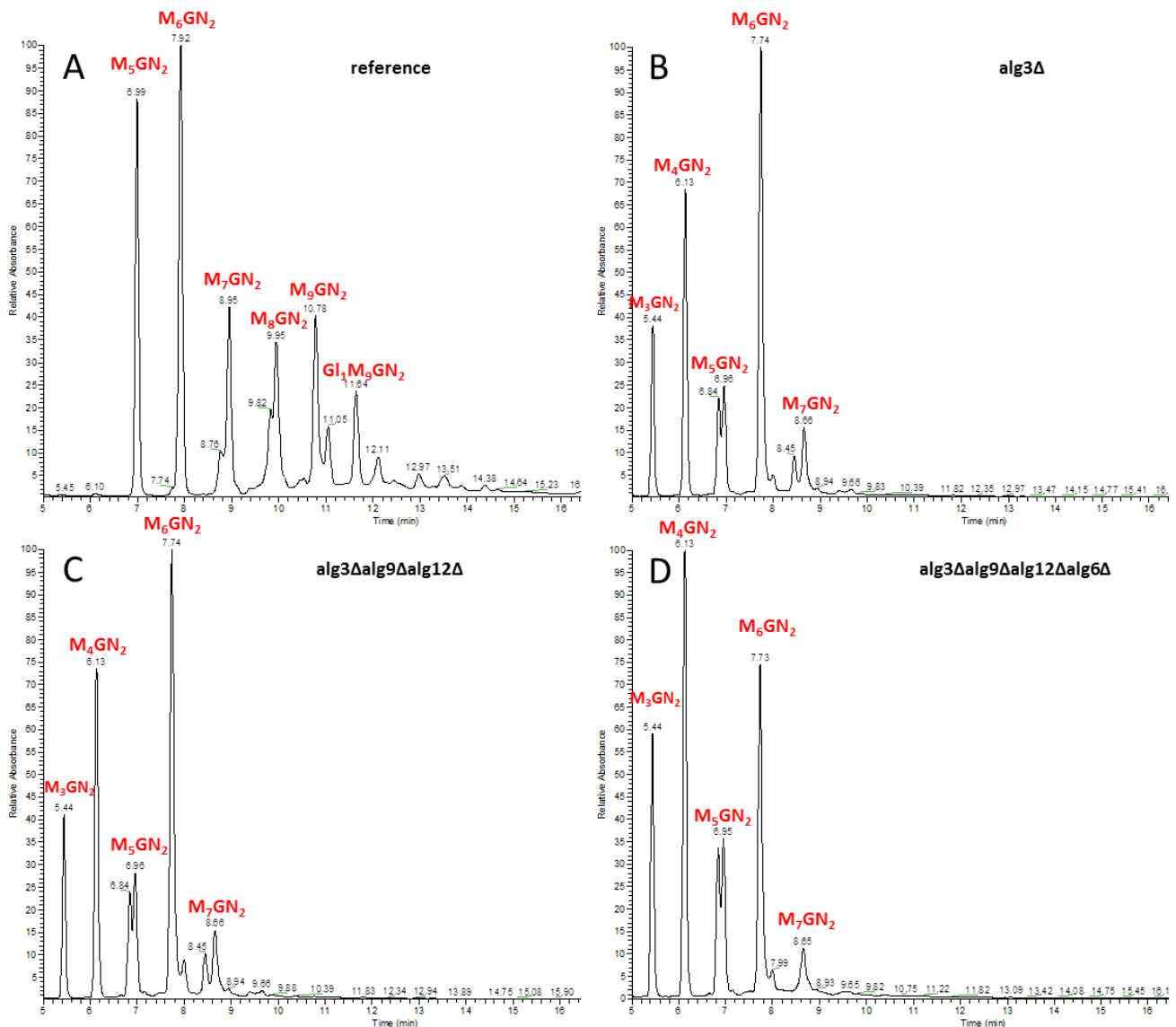


Figure 3.3: N-glycan analysis of purified β -glucosidase. Deletion of mannosyltransferases shifted the glycan profile toward a lower mass. Abbreviations: M: Mannose; GN: N-acetylglucoseamine; Gl: Glucose.

Deletion of *alg6* removed peak with glucose cap

Alg6 catalyzes the addition of the first glucose to the $Man_9GlcNAc_2$ -PP-Dol precursor before transfer of the glycan from the dolichol anchor to the protein (Reiss et al., 1996). Deletion of this gene, should thus remove any residual (capping) glucose left on the glycan, which is transferred to the Golgi. The drawback of this deletion is a less efficient transfer of the glycan from the dolichol anchor to the protein (De Pourcq et al., 2012) resulting in reduced glycosylation occupancy. The putative *alg6* gene, *AN4864*, was identified in the same manner as the other mannosyltransferases using *S. cerevisiae* Alg6p (YOR002W) as query. Analysis of the glycans revealed that one of the peaks with a mass corresponding to $Man_7GlcNAc_2$ disappeared, indicating that this peak probably consisted of $GlcMan_6GlcNAc_2$ (see Figure 3.3D). In addition,

the ratio of GlcMan₃₋₅GlcNAc₂ was increased in the *alg3Δalg9alg12Δalg6Δ* mutant. The increased ratio of GlcMan₃₋₅GlcNAc₂ could be due to the removal of glucose capped structures, which are not recognized by the mannosidases. To verify how many of the glycans observed in the reference with a glucose cap and also to investigate which effect the *alg6Δ* alone has on the glycan profile, the *alg6* gene was deleted in the reference strain. The glycan analysis showed that glycans with masses over Man₉GlcNAc₂ were not observed in the deletion strain (see Figure 3.4B). This suggests that some of the observed structures were likely due to some incompletely processed glycan structures being transported to the Golgi.

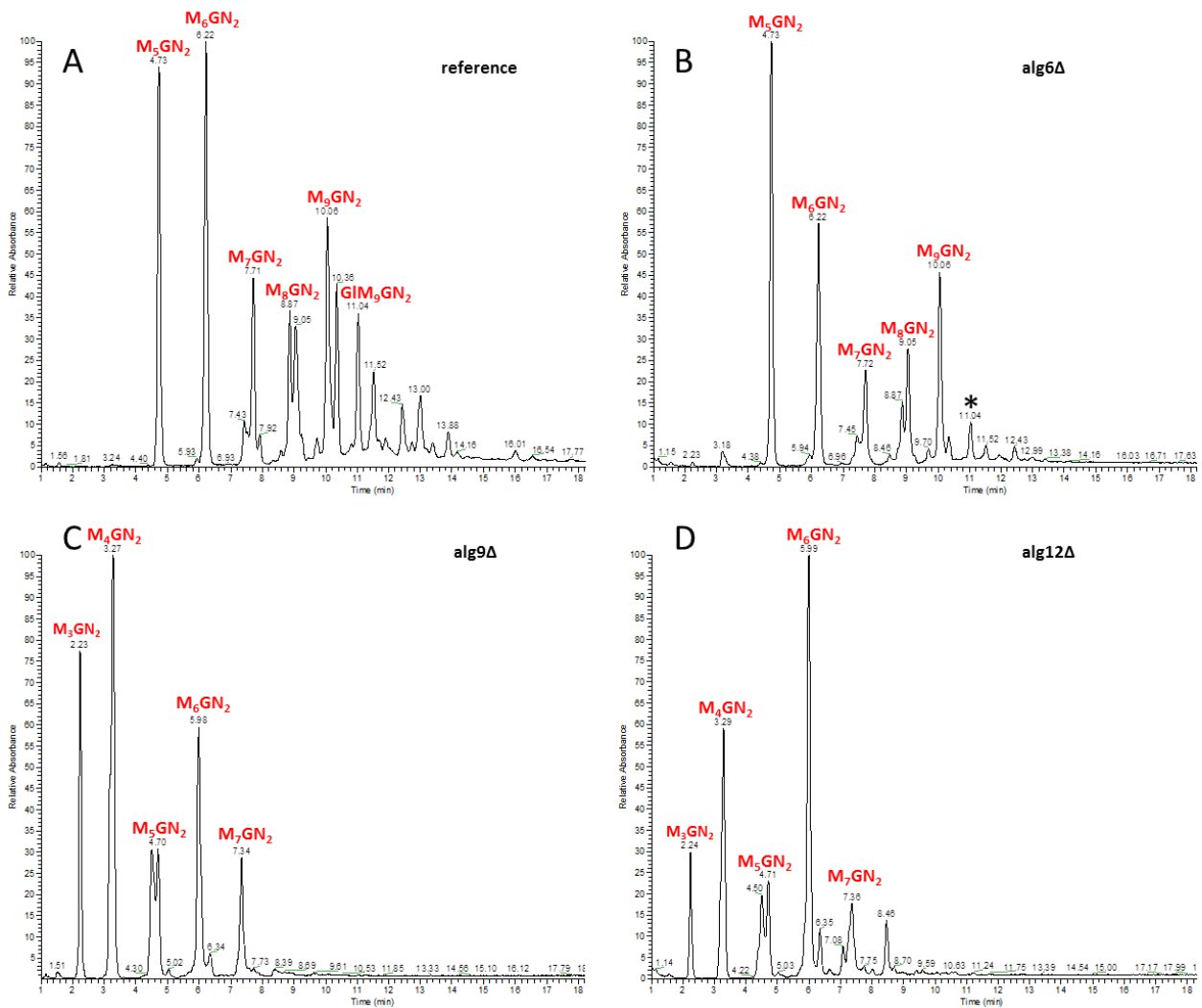


Figure 3.4: N-glycan analysis of purified β -glucosidase. (A) Reference strain. (B) Deletion of *alg6* removes peak corresponding to a glycan structure capped with glucose. (C) and (D) Deletion of *alg9* and *alg12* resulted in a shift of the glycans toward a lower mass, respectively. Abbreviations: M: Mannose; GN: N-acetylglucosamine; Gl: Glucose. * No mass was measured in the MS.

Single deletions of *alg9* and *alg12* generate the truncated $\text{Man}_{3-5}\text{GlcNAc}_2$

In order to investigate the effect of the single deletions of the putative *alg9* and *alg12* as well as if the intermediates, consisting of $\text{Man}_{3-5}\text{GlcNAc}_2$ could be generated by deleting these individual genes in the ER, single deletion mutants were generated. The glycan analysis showed that the glycosylation profile of the *alg9* Δ and *alg12* Δ mutant strains was shifted towards lower glycans consisting of $\text{Man}_{3-7}\text{GlcNAc}_2$ and $\text{Man}_{3-8}\text{GlcNAc}_2$ respectively (see Figure 3.4C & D). This suggests that mannosidases present in the ER or Golgi were able to trim the truncated structure generated by the deletion of *alg9* and *alg12*, although there was an increased variance in the extent of trimming between N-glycan samples from different batches of *alg9* Δ and *alg12* Δ than between batches of *alg3* Δ (data not shown). This suggests that the mannosidases present in the ER and Golgi were able to trim other bonds than α -1,2 bonds on the truncated glycan precursor.

The localization of Alg9 was investigated by generating a fusion protein by fusion of mRFP to the C-terminal of Alg9. Fluorescence microscopy gave the same pattern as Alg3 indicating that Alg9 is co-localized with Alg3, and it is very likely that they both localize to the ER, see Figure 3.5.



Figure 3.5: Localization of ALG9-mRFP fusion protein.

Summary and perspectives

This study has demonstrated that the mannosyltransferase activity responsible for adding mannose to the truncated $\text{Man}_5\text{GlcNAc}_2$ generated by the deletion of *alg3* is not due to the known ER localized mannosyltransferases involved in N-glycan synthesis. Hence, the addition of mannose to the $\text{Man}_5\text{GlcNAc}$ likely takes place in the Golgi. It seems possible that the $\text{Man}_5\text{GlcNAc}$ is trimmed down to $\text{Man}_3\text{GlcNAc}$ and built up again to $\text{Man}_5\text{GlcNAc}_2$ or higher glycan structures. Based on this, two approaches can be employed to generate more of the $\text{Man}_3\text{GlcNAc}$. The first approach is to identify the mannosyltransferases in the Golgi and subsequently delete them to remove further addition of mannose. This is similar to the work

done by Parsaie Nasab and co-workers, where they showed that the deletion of a mannosyltransferase in *S. cerevisiae* resulted in a less heterogeneous glycan pattern (Parsaie Nasab et al., 2013). On the other hand their study also suggested that a combined deletion of mannosyltransferases is needed to completely abolish the unwanted mannosyltransferase activity. This might also be the case in *A. nidulans*. The second approach is to identify the mannosidase responsible for the trimming of the $\text{Man}_5\text{GlcNAc}_2$. Identifying this enzyme would provide further insight; first pinpointing the enzyme with the desired activity, and secondly, demonstrate whether trimming and rebuilding of the $\text{Man}_5\text{GlcNAc}$ takes place. Using these approaches the generation of more homologous glycan of $\text{Man}_3\text{GlcNAc}$ structure can be obtained and can thereby facilitate the further humanization of the glycosylation pathway in *A. nidulans*.

Materials and methods

Strains and media

The *A. nidulans* strain IBT 29539 (*argB2*, *pyrG89*, *veA1*, *nkuAΔ*) (Nielsen et al., 2008) was used for strain construction. A list of strains used in this study is provided in Table 3.3. Plasmids were propagated in *E. coli* strain DH5α. *A. nidulans* strains were grown on minimal medium (MM) containing 1% glucose, 0.001 % thiamine, 0.1 % trace element solution and 5 % nitrate salts solution. For solid medium 2 % agar was added. Nitrate salts solution (per Liter): 120 g NaNO_3 , 10.4 g KCl, 10.4 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 30.4 g KH_2PO_4 . Trace element solution (per Liter): 0.4 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.04 g $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$, 0.8 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.8 g $\text{MnSO}_4 \cdot 2\text{H}_2\text{O}$, 0.8 g $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 8 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$. MM was supplemented with 10 mM uridine (Uri), 10 mM uracil (Ura) and/or 4 mM L-arginine (Arg), when necessary. Solid medium containing 5-fluoroorotic acid (5-FOA) was made as MM+Ura+Uri+Arg supplemented with 1.3 mg/mL %FOA. Induction of Tet-on promoter system (Meyer et al., 2011a) was achieved by addition of doxycycline. For shake flask cultivations spores were harvested in distilled water 500 mL shake flasks (without baffles) were inoculated with 10^7 spores/mL 30°C and 150 rpm. Restriction enzymes and buffers were from New England Biolabs.

PCR and vector construction

All PCR products were amplified using proofreading PfuX7 polymerase (Nørholm, 2010) and either HF or Phire PCR buffer (Thermo Scientific) in 35 reaction cycles with 60°C annealing temperature. All fragments relating to *A. nidulans* were amplified from *A. nidulans* IBT 29539 gDNA. mRFP was amplified from sequence derived from pSK800 (Toews et al., 2004). The list of all primers used in this study is found in Table 3.1.

The deletion of putative mannosyltransferase genes were performed using bipartite gene-targeting substrates (Nielsen et al., 2006). Each of the bipartite substrates consists of a targeting sequence and part of the marker sequence. The targeting sequence and marker sequence were individually amplified by PCR using primers with adaptamers (Erdeniz et al., 1997). The targeting sequence and marker sequence were subsequently fused together by PCR to generate the targeting substrate. The *AFpyrG* marker sequence was amplified from pDEL2 (Nielsen et al., 2008) using 5A and 2K for the first part of *AFpyrG* and 4Q and 2B for the last part. Specific combinations of primers used for generating substrates are in Table 3.2.

To construct the plasmid for overexpression of the putative β -glucosidase *AN1804* was amplified from gDNA with primers DC135 and DC136. Primer DC136 contains the sequence for adding a C-terminal 6•His-tag to *AN1804*. The PCR fragment was inserted into pU1111-5-ccdB by USER cloning. The plasmid was prepared for USER cloning by digesting with *Asi*I and *Nb.Bts*I. The cloning procedure was as described in (Nour-Eldin et al., 2006). Plasmids were verified by restriction analysis and linearized by digestion with *Swa*I prior to transformation.

The Alg3-RFP and Alg9-RFP fusion construct were prepared by amplifying *alg3* and *alg9* with DC211-DC212 and DC213-DC214, respectively. *rfp* was amplified from pU2110-1-*rfp* using primers C83 and C54. The PCR fragment was inserted into of pU2311-1-ccdB by USER cloning. Plasmid was prepared as previously described.

***A. nidulans* strain construction**

Protoplastation and transformation of *A. nidulans* were performed as described (Johnstone et al., 1985; Nielsen et al., 2006) using *AFpyrG* or *argB* as a selectable marker. All transformants were verified with PCR analysis using spores as the source of DNA. At the start of the PCR the samples were incubated 20 minutes at 98°C in order to liberate the DNA from the cells. This was followed by a touchdown PCR program with annealing temperatures from 64°C to 55°C. The reactions were carried out in 35 reaction cycles. The spores were transferred to the PCR mix by gently touching a colony with a pipette tip and transferring the spores to the PCR tube.

Strains transformed with *AFpyrG* were subjected to counter selection on MM+Ura+Uri+Arg +5-FOA in order to recycle the *AFpyrG* marker, hence, allowing the use of the marker for the subsequent deletions of transferases (*alg9*, *alg12* and *alg6*).

β -glucosidase purification

After 48 hours of cultivation the culture medium was separated from the mycelia by filtration through two layers of miracloth. The culture medium was concentrated by using a centrifugal unit (Amicon Ultra-15

Centrifugal Filter Unit with Ultracel-10 membrane). The His-tagged protein was purified using the His SpinTrap kit (GE Healthcare). Purified proteins were loaded on SDS-PAGE (Novex NuPAGE 4-12% Bis-Tris gel from Life Technologies) according to the instructions of the manufacturer and protein concentration measured by Coomassie Bradford assay (Thermo Scientific).

Fluorescence microscopy

MM agar slides were prepared by pipetting 1 ml MM with agar on a slide. MM agar slides were inoculated with spores and grown O/N at 30°C in petri dishes. Live cell images were captured with a cooled Evolution QEi monochrome digital camera (Media Cybernetics Inc.) mounted on a Nikon Eclipse E1000 microscope (Nikon). Images were captured using a Plan-Fluor x100, 1.30 numerical aperture objective lens. The illumination source was a 103-watt mercury arc lamp (Osram). The fluorophore RFP was visualized using a band pass RFP filter (EX545/30, EM620/60 combination filter; Nikon).

N-glycan analysis

N-glycans from β glucosidase were released and fluorescence labeled with GlykoPrep Rapid N-Glycan kit from ProZyme using 2-AB as fluorescence label. Labeled N-glycans was analysed on LC-MS.

LC-MS System was a Thermo Ultimate 3000 HPLC with fluorescence detector coupled on-line to a Thermo Velos Pro Iontrap MS. Separation was done on a Waters BEH Glycan column 100mm x 2.1, 1.7 μ m and Solvents: A: 100% Acetonitrile, B: 50mM Ammonium Formate, pH 4.4 adjusted with Formic acid and filtered 0.2 μ m. Separation gradient from 39% buffer to 47% over 16min. at 0.5mL/min flowrate. Fluorescence detector set to High power lamp and 360nm excitation, 428nm emission. MS settings: Fullscan: 700-2000m/z, source fragmentation 60V, polarity Negative.

Table 3.1: List of primers used in this study

Name	Sequence ^a
B11	CCGCGACCATCACTGAAC
B12	CATCCGGCCAGGCTACTTACC
B13	gatccccgggaattgccatgCGGAGTATCGGAGTATGCTGGC
B14	aattccagctgaccaccatgGCCAGGTGTTAGGCGTCC
B15	GGATTGATCCACGAGATCACCG
B16	GCATCCAAGGGTCATGCG
B23	GGTGTAGCCATCTTCGGGTACG
B24	GTGAGCCAGCCTCGAGGAC
B25	gatccccgggaattgccatgCTGGACGTAAAGGATCGTCCCTC
B26	aattccagctgaccaccatgGTCTCGCTGTTGCACGTACC
B27	CATGAGAGCGAGGTCGTCTCTG

B28	CCAAGATACCACGTCCGCATC
ØØ9	CGCTACAGTCCGGTACCTATG
ØØ10	gatccccgggaattgccatgCCTCCACATGATGTGCGC
ÅÅ2	CGGACTCGGATATTGACAGGG
ÅÅ3	CCGTACTGCTTGATGCCAAG
B85	CTGGAGGCATCCTTCATCTC
B86	CTTCACCTCCTTCTTGACCG
B87	gatccccgggaattgccatgGCCTATAACGGAGTATGTGAG
B88	aattccagctgaccaccatgCGTGGTCCGACATGTATAG
B89	CTCAATAGTCGCAAGCGG
B90	CAGCCGAGTCCAATTCTAG
B91	GGTCGAGTCATGGCAAGTG
DC81	aattccagctgaccaccatgCTGCGCTTAAGTGTATTATGTTGGTC
5A	TGGATAACCGTATTACCGCC
2B	TGCCAAGCTTAACGCGTACC
2K	GGAAGAGAGGTTACACC
4Q	TGATACAGGTCTCGGTCCC
DC211	AGAGCGAUATGGCATTGACAGACCTTGTG
DC212	AGGAGGCCAUCTGAACGTGTTGCTTTGCTTTTGG
DC213	AGAGCGAUATGCCTCGAAAAGGAATTGAGG
DC214	AGGAGGCCAUTACTAACTCCGACTCAGAGTCC
DC135	AGAGCGAUATGAGGGTTGATTCAACAGTTCTG
DC136	TCTGCGAUTCAATGATGATGATGATGATGACCGGAGCCGTTATCCTCGTACTCCAGGC
C83	ATGGCCTCCUCCGAGGACG
C54	TCTGCGAUTTAGGCGCCGGTGGAGTG

^aLower case letters refer to tails used for the formation of bipartite substrate

Table 3.2. Primers used for the construction of deletion strains and primers used for PCR verification of gene deletion

Gene		Bipartite substrate ^a		Verification
		up	dw	
<i>alg3Δ</i>	<i>AN0104</i>	B12+B13	B14+B15	B11&B16
<i>alg6Δ</i>	<i>AN4864</i>	B86+B87	B88+B89	B85&B90
<i>alg9Δ</i>	<i>AN10118</i>	B24+B25	B26+B27	B23&B28
<i>alg12Δ</i>	<i>AN3588</i>	ØØ9+ØØ10	DC81+ÅÅ2	B91/ÅÅ3

^aUp and dw fragment were fused to pyrG marker up and dw fragments. 5A/2K and 2B/4Q were used for the amplification of the pyrG up and dw fragments respectively

Table 3.3. List of strains used in this study

Strain	Genotype	Source
IBT 29539	<i>argB2, pyrG89, veA1, nkuAΔ</i>	(Nielsen et al., 2008)
Reference	<i>argB2, pyrG89, veA1, nkuAΔ, IS5::PgpdA::AN1804::TtrpC::argB</i>	
alg3Δ	<i>argB2, pyrG89, veA1, nkuAΔ, AN0104Δ, IS5::PgpdA::AN1804::TtrpC::argB</i>	This study
alg6Δ	<i>argB2, pyrG89, veA1, nkuAΔ, AN4864Δ, IS5::PgpdA::AN1804::TtrpC::argB</i>	This study
alg9Δ	<i>argB2, pyrG89, veA1, nkuAΔ, AN10118Δ, IS5::PgpdA::AN1804::TtrpC::argB</i>	This study
alg12Δ	<i>argB2, pyrG89, veA1, nkuAΔ, AN3588Δ, IS5::PgpdA::AN1804::TtrpC::argB</i>	This study
alg3Δalg9Δalg12Δ	<i>argB2, pyrG89, veA1, nkuAΔ, AN0104Δ, AN10118Δ, AN3588Δ, IS5::PgpdA::AN1804::TtrpC::argB</i>	This study
alg3Δalg6Δalg9Δalg12Δ	<i>argB2, pyrG89, veA1, nkuAΔ, AN0104Δ, AN4864Δ, AN10118Δ, AN3588Δ, IS5::PgpdA::AN1804::TtrpC::argB</i>	This study
Alg3-RFP	<i>argB2, pyrG89, veA1, nkuAΔ, IS1::Ptet-on::AN0104-rfp::TtrpC::AFpyrG</i>	This study
Alg9-RFP	<i>argB2, pyrG89, veA1, nkuAΔ, IS1::Ptet-on::AN10118-rfp::TtrpC::AFpyrG</i>	This study

4 Conclusion and perspectives

The work presented in thesis has demonstrated the use of *A. nidulans* as an expression platform for studying secondary metabolism as well as expanding the repertoire of glycoproteins, which can be produced in filamentous fungi.

A. nidulans was used as host for heterologous expression of the geodin gene cluster from *A. terreus*. The many genetic tools available for *A. nidulans* make it a suitable host for heterologous gene expression. For example development of strains with impaired NHEJ significantly increases the gene-targeting efficiency and facilitates easier screening for correct transformants. A method based on USER mediated assembly of PCR products was developed to transfer the putative geodin cluster, spanning 25 kb, to a defined locus, *IS1*, in *A. nidulans* through two sequential gene-targeting events. The selection marker was replaced with a second marker after the first integration event facilitating the recycling of the previous marker. Thus, there is no limit to number of gene-targeting events, which can be carried out and the transferable gene cluster size seems to be unlimited. Successful production of geodin from the transferred cluster substantiated that the gene cluster was indeed encoding the genes necessary for geodin production. Furthermore, the functions of three genes; *gedL*, *gedC* and *gedR* were shown to encode the cluster specific TF, PKS and halogenase, respectively. This method is an efficient and versatile way to transfer cryptic SM clusters from other fungi to *A. nidulans* to facilitate easier elucidation of SM biosynthetic pathways and link genes to products.

Despite placing the TF from the geodin gene cluster under the control of the strong constitutive *gpdA* promoter only small amounts of geodin was produced, when the monodictyphenone (*mdp*) pathway was deleted. As the *mdp* pathway shares several steps with the geodin pathway it was assumed that natively produced intermediates from the *mdp* cluster could be converted to geodin, resulting in production of higher amounts of geodin compared to the strain without the *mdp* cluster. This indicated that though the heterologously expressed genes were functional, as indicated by geodin production in the *mdpΔ* strain, they were not as efficient or expressed in as high a level as the homologous native genes. An alternative to the approach developed here is to place each gene in the cluster under the control of a strong promoter, thus ensuring a high expression of each gene independently of the TF. This approach requires more sequential gene-targeting events, is more time consuming and relies on the correct annotation of all genes in the cluster prior to integration in the host. But if a higher titer of product is desired, this might prove to be a better approach to use.

In addition to the possibility of low expression of genes/activity of enzymes lower production of geodin could be due to limited access to substrate. Since it was hypothesized that the intermediates of the monodictyphenone pathway could be converted to geodin the localization of the PKS from both pathways were investigated by fluorescence microscopy. There were indications that the two PKSs co-localized, possibly in organelles related to the peroxisomes. Co-localization to specific compartments will facilitate easier exchange of intermediates and explain why higher production of geodin was achieved when the *mdp* cluster was intact. Gaining insight to the spatial distribution and localization of the remaining enzymatic steps of both pathways may identify points where they differ. If the heterologous enzyme is localized to a compartment, where the substrate is not readily available, it will likely result in less efficient geodin production.

In this thesis, a different approach to activate silent clusters was employed as it was investigated whether heterologous expression of putative regulators from *A. niger* would be able to influence secondary metabolite production in *A. nidulans*. Seven regulators (six TFs and one putative histone demethylase) were placed under the *gpdA* promoter and integrated in *IS1* in *A. nidulans*. Interestingly, after screening on several media the expression of one of the regulators, a putative TF, named *smrA* in this study, induced synthesis of insect juvenile hormone-III and methyl farnesoate, when grown under high salt concentration. It was also discovered that feeding by *Drosophila melanogaster* larvae induced synthesis of insect juvenile hormone in *A. nidulans*. The precise function of the juvenile hormone was not determined, as the feeding experiments gave no clear indications as to whether or not this was an antagonistic interaction. Nevertheless, this indicates that, in addition to co-cultivation with microorganisms, insect-fungus confrontation systems can be used for the activation of SM pathways as well as provide clues to the biological function of these metabolites. Furthermore, this opens up an avenue where fungi, as *A. nidulans*, can be used as cell factories for the production of juvenile hormones, which are considered to be promising insecticides (Marrs, 2012).

Production of glycosylated therapeutic proteins in filamentous fungi is hampered by the difference in glycan structures, which are attached to this protein. To overcome this hurdle, an approach to generate a homogenous pool of glycan precursors which can function as building blocks for human-like glycan structures was utilized. Inspired by the work done by (Kainz et al., 2008), three putative mannosyltransferases (Alg3, Alg9 and Alg12), which are responsible for building the glycan precursor in the ER, were deleted in *A. nidulans* in order to generate a pool of glycans consisting of Man₃₋₅GlcNAc₂. This deletion study excluded the involvement of ER localized transferases in the extension of the structure. This study also showed that two isoforms of Man₅GlcNAc₂ are present in the deletion strains, suggesting that

the truncated $\text{Man}_5\text{GlcNAc}_2$ is trimmed to $\text{Man}_{3-4}\text{GlcNAc}_2$ and elongated to $\text{Man}_{5-7}\text{GlcNAc}_2$ by Golgi localized mannosyltransferases. Furthermore, truncated structures of the glycan precursor generated by the deletion of *alg9* and *alg12* could also be trimmed to $\text{Man}_{3-4}\text{GlcNAc}_2$, indicating that these deletions also can generate the desired building block for further modifications. Deletion of the putative mannosyltransferases localized in the Golgi as well as identifying the mannosidase responsible for the trimming of the glycan structure will facilitate the generation of a more homogenous pool of glycan precursors, and this will be the aim of future work. Utilizing *A. nidulans* as a model to engineer human-like glycosylation will lay the groundwork for the humanization of the glycan pathway of other *Aspergilli* cell factories paving the way for production of therapeutic proteins in these systems.

5 Bibliography

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